Universidad Pública de Navarra

Nafarroako Unibertsitate Publikoa

Facultad de Ciencias de la Salud

Immune reconstitution against Cytomegalovirus in allogeneic haematopoietic stem cell transplant recipients

Miriam Ciáurriz Gortari

2015

Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

Facultad de Ciencias de la Salud

Immune reconstitution against Cytomegalovirus in allogeneic haematopoietic stem cell transplant recipients

Memoria presentada por Dª Miriam Ciáurriz Gortari para aspirar al grado de Doctor por la Universidad Pública de Navarra.

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Oncohematología y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, … de Junio de 2015.

Dr. Eduardo Olavarría López-Aróstegui

Dra. Eva Bandrés Elizalde

Dra. Natalia Ramírez Huerto

A mis padres, José Luis y María,
a mis hermanas, Paula y Marta,
y a Javier, por su amor y cariño
incondicional.

AGRADECIMIENTOS

A la Universidad Pública de Navarra por darme la posibilidad de seguir formándome como investigadora y a Navarrabiomed-Fundación Miguel Servet por haberme dado la oportunidad de hacer el doctorado en su centro.

A mi director de tesis, el Dr. Eduardo Olavarría, creador de este proyecto, y a mis codirectoras Dra. Eva Bandrés y Dra. Natalia Ramírez, por su apoyo durante estos 4 años.

Al grupo de investigación de Oncohematología por darme la oportunidad de realizar la tesis doctoral y acogerme dentro de sus proyectos de investigación.

Al laboratorio de Hematología del Complejo Hospitalario de Navarra por proporcionarme las muestras.

A Lorea, que me enseñó a dar mis primeros pasitos en investigación, por todo el apoyo que me ha dado incluso en la distancia, por sus consejos, las largas horas de trabajo en el laboratorio, las interminables conversaciones de skype y su amistad.

A Cris, Estela, Mercedes y Amayica, por todos los buenos momentos pasados con vosotras, en el labo o en el despacho, por aguantarme los momentos de estrés, por los cafés, todos menos los de *'luis'* que esos me quitaban mucho tiempo, por vuestro buen humor y consejos, y por escucharme.

A Natalia López, Quique y Jokin, por escucharme cuando necesitaba desconectar y darme siempre buenos consejos. A Xabi, por ayudarme con la estadística y aconsejarme.

I would also like to thank Professor Paul Moss for giving me the opportunity to undertake research in his group during one year of my PhD. I also owe a big thank you to Dr. Annette Pachnio, for teaching me flow cytometry, cell culture and for her constant support and help. Thank you to Kirsty, Helen and Louise, for all the laughs and time we spent together.

A Sara, Manolo y David, mi familia en Birmingham, por todos los buenos momentos que hemos pasado juntos, por los consejos, por los momentos de locura, unos más que otros, por las pints, bueno o cider!

A Bea, María, Raquel, Cris, Olga y Ana, por todo el tiempo pasado con vosotras y el que nos queda, por el apoyo y los ánimos dados.

A Edurne, Marta, Marina y Belén. Por todos los momentos compartidos, los buenos y malos, los divertidos y los tristes, y por todos los que nos quedan, porque siempre estáis

ahí, por comprenderme este último año y los ánimos dados. Por ayudarme a desconectar de las largas horas de trabajo.

A mis padres, por todo lo que me han enseñado y todo lo que me han dado, por escucharme, guiarme y quererme tal como soy. A mis hermanas, Paula y Marta, porque siempre estáis ahí, y los buenos momentos no nos los quita nadie. Y al resto de mi familia, porque me apoyáis año tras año. Os quiero!

A Javier, por estar a mí lado. Gracias por apoyarme durante todos estos años, por tu ánimo y tu comprensión. Por este último año, por los 'hoy no puedo que tengo que escribir', 'un poco más tarde que estaba concentrada'. Por escucharme y aconsejarme y hacer que no tirara la toalla cuando todo lo veía negro. Te quiero!

MUCHAS GRACIAS A TODOS

ABBREVIATIONS

ALL	Acute Lymphoblastic Leukaemia
Allo-HSCT	Allogeneic haematopoietic stem cell transplantation
AML	Acute Myeloid Leukaemia
APCs	Antigen Presenting Cells
APC	Allophycocyanin
ATG	Anti-thymocyte globulin
BM	Bone Marrow
Bu	Busulfan
CD	Cluster differentiation
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia
CMV	Human Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CXCR	Chemokine Receptor
Cs-A	Cyclosporine A
Cy	Cyclophosphamide
DNA	Deoxyribonucleic acid
DNAemia	DNA-viremia
EBMT	European Bone Marrow Transplantation
EBV	Epstein-Barr Virus
EBV-LCL	EBV-transformed B-Lymphoblastoid Cell Lines
ELISA	Enzyme-linked-immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
ER	Endoplasmic reticulum
Flu	Fludarabine
FSC	Forward Scatter
G-CSF	Granulocyte-Colony Stimulating Factor
GCV	Ganciclovir
GMP	Good Manufacturing Practice
GvHD	Graft versus Host Disease
aGvHD	Acute graft versus host disease
cGvHD	Chronic graft versus host disease
gB	Glycoprotein B

HL	Hodgkin lymphoma
HLA	Human Leukocyte Antigen
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplantation
ICS	Intracellular Cytokine staining
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
i.v.	Intravenous
MA	Myeloablative
MDS	Myelodysplastic syndrome
Mel	Melfalan
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MSC	Mesenchymal stem cell
MMF	Mycophenolate mofetil
MTX	Methotrexate
o/n	Overnight
PAMPs	Pathogen-associated molecular patterns
p.o.	Oral
PBMCs	Peripheral Blood Mononuclear Cells
PBSCs	Peripheral Blood Stem Cells
qPCR	Quantitative Polymerase Chain Reaction
PD-1	Programmed cell death-1
PDN	Prednisone
PM	Pentamer
Pp65	Phosphoprotein 65
RIC	Reduced Intensity Conditioning
RT	Room Temperature
SNPs	Single Nucleotide Polymorphisms
SSC	Side Scatter
ST	Streptamer
TAP	Transporter associated with antigen processing

TBI	Total Body Irradiation
T_{CM}	Central Memory T cells
TCR	T Cell Receptor
T_{CM}	Central Memory T cell
T_{EM}	Effector Memory T cell
T_{EMRA}	$CD45RA^{+}$ Effector Memory T cell
Th	T helper
TLR	Toll-like receptor
TNFα	Tumour Necrosis Factor alpha
Tregs	Regulatory T cells
UCB	Umbilical Cord Blood
URD	Unrelated donor
VGC	Valganciclovir

INDEX

I) INTRODUCTION

1 General introduction to immunology

The immune system is a network of cells, tissues, and organs that work together to protect the body from infection. The overall function of the immune system is to prevent or limit infection.

The immune response is generally divided into innate and adaptive immunity:

Innate immune system provides immediate defence against infection. Innate immunity recognizes and responds to pathogens in a generic way, but it does not confer long-lasting or protective immunity to the host. This response depends on a group of proteins and phagocytic cells that recognize conserved features of pathogens and become quickly activated to help destroy invaders called pathogen-associated molecular patterns (PAMPs). Innate immune cells express genetically encoded receptors, called Toll-like receptors (TLRs), which recognize general PAMPs (1). Collectively, these receptors can broadly recognize viruses, bacteria, fungi, and even non-infectious problems. However, they cannot distinguish between specific strains of bacteria or viruses. There are numerous types of innate immune cells with specialized functions, including neutrophils, eosinophils, basophils, mast cells, monocytes, dendritic cells (DCs), macrophages and natural killer (NK) cells. Their main feature is the ability to respond quickly and broadly when a problem arises, typically leading to inflammation. These cells release cytokines that help to destroy the pathogen in other cells by inducing a series of distinct processes. Cytokines are important in the activation, proliferation, differentiation and chemotaxis of immune cells (2). Innate cells are critical for host defence, and disorders in innate cell function may cause chronic susceptibility to infection. When the invader escapes or survives the innate immune system, the adaptive response is activated.

Adaptive immunity occurs later, as it relies on the coordination and expansion of specific immune cells. Therefore, the adaptive immune responses are highly specific to a particular pathogen that induced them. The function is to destroy invading pathogens and any toxic molecules they produce. Because these responses are destructive, it is crucial that they be made only in response to molecules that are foreign to the host and not to the molecules of the host itself. The ability to distinguish what is foreign from what is self in this way is a fundamental feature of the adaptive immune system. Any substance capable of eliciting an adaptive immune response is referred to as an antigen. Antigens are usually

proteins that are too large to bind as a whole to any receptor so only specific segments that form the antigen, bind with a specific receptor. Such segments are called epitopes. A conformational epitope is a sequence of subunits (usually amino acids) composing an antigen that come in direct contact with a receptor of the immune system. Adaptive immune responses are carried out by white blood cells called lymphocytes. There are two broad classes of such responses: antibody responses and cell-mediated immune responses that are carried out by different classes of lymphocytes, called B cells and T cells, respectively (3). In this thesis we will focus on T cell lymphocytes.

1.1 T-cell lymphocytes

T cell lymphocytes are antigen-specific and can help eliminate pathogens that reside inside host cells. T cells maturate in the thymus and then T lymphocytes migrate to peripheral lymph organs. First, T cells are activated by foreign antigen to proliferate and differentiate into effector cells only when the antigen is displayed on the surface of antigen-presenting cells (APC). T cells recognize through the T cell receptor (TCR), short peptides of antigens (8-15 amino acids) that have been partly degraded inside the APC. The peptide fragments are then carried to the surface of the APC on special molecules called major histocompatibility complex (MHC). Once activated, effector T cells act only at short range, either within a secondary lymphoid organ or after they have migrated into a site of infection. They interact directly with another cell in the body, which they either kill or signal in some way.

The human MHC is coded by the human leukocyte antigen (HLA) system. The HLA system is located on chromosome 6 and it is divided into three regions (Figure 1A). The class I region contains the classical HLA-A, HLA-B and HLA-C genes that encode the heavy chains of class I molecules. The class II region consists of a series of subregions, each containing A and B genes encoding α and β chains, respectively. The DR gene family consists of a single DRA gene and up to nine DRB genes. HLA-DR specificities are determined by the polymorphic DRβ1 chains encoded by DRB1 alleles. The DQ molecules are formed by DQA1 and DQB1 gene products and DPA1 and DPB1 products associate to form DP molecules. The class III region does not encode HLA molecules, but contains genes for the complement components (C2, C4, factor B), tumour necrosis factors (TNFs) and some others (4).

Class I molecules consist of a heavy chain formed by three extracellular domains (α_1, α_2 and α_3), a transmembrane region and an intracytoplasmic domain, and a noncovalently bound extracellular β_2-microglobulin (β_2m) (Figure 1B). The products of the class II genes DR, DP and DQ are heterodimers of two noncovalently associated chains, α and β. An extracellular portion composed of two domains (α_1 and α_2, or β_1 and β_2) is anchored on the membrane by a short transmembrane region and cytoplasmic domain. The HLA system is known to be the most polymorphic in humans. This polymorphism is not evenly spread throughout the molecule, but is clustered in the antigen binding groove.

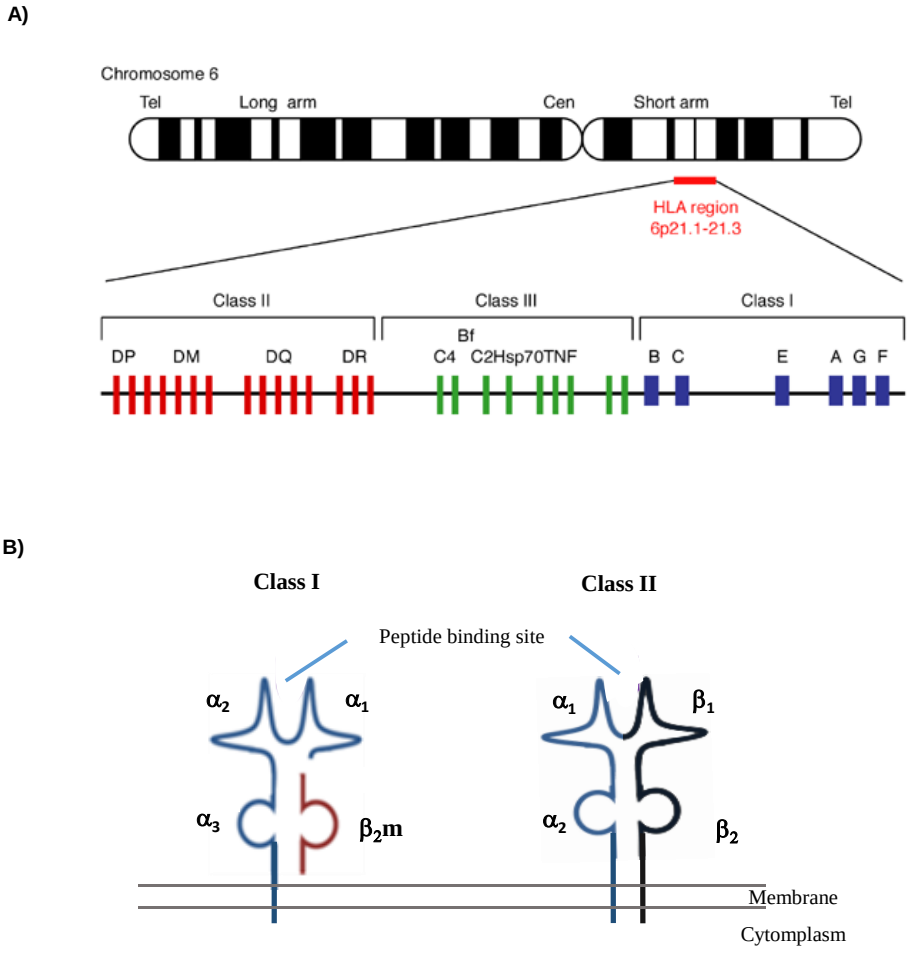

Figure 1. Gene map of the HLA region. Adapted from *Cambridge University Press* (A). Schematic diagram of HLA class I and class II molecules (B).

MHC proteins function as carriers to present antigens on cell surfaces. MHC class I proteins are essential for presenting viral antigens and are expressed by nearly all cell types, except red blood cells. Any cell infected by a virus has the ability to signal the

problem through MHC class I proteins. MHC class II proteins are generally only expressed by B lymphocytes, DCs and macrophages. MHC class II antigens are varied and include both pathogen- and host-derived molecules (5).

The TCR are complexes composed of two variable chains responsible for antigen recognition and CD3 subunits and ζ chains, both of which initiate signalling cascades. The variable chains of most lymphocytes are the product of random rearrangements of the α and β genes (the large repertoire of the receptors of T cells). Some T lymphocytes contain, as variable chains, the products of the γ and δ genes, called γδ T cells (6). This random generation of receptors allows the immune system to respond to unforeseen problems and also explains why T cells are highly specific and, upon re-encountering their specific pathogen, can immediately induce a neutralizing immune response.

There are two main classes of T cells that present the TCR as well as either CD8 or CD4 on their surfaces. Both CD8 and CD4 are important for the recognition of the MHC presenting the antigen to the T cells. Cytotoxic $CD8^+$ T cells (CTLs) directly kill cells that are infected with a virus or some other intracellular pathogen whereas $CD4^+$ T cells help stimulate the responses of other cells (mainly B cells and cytotoxic cells) (Figure 2).

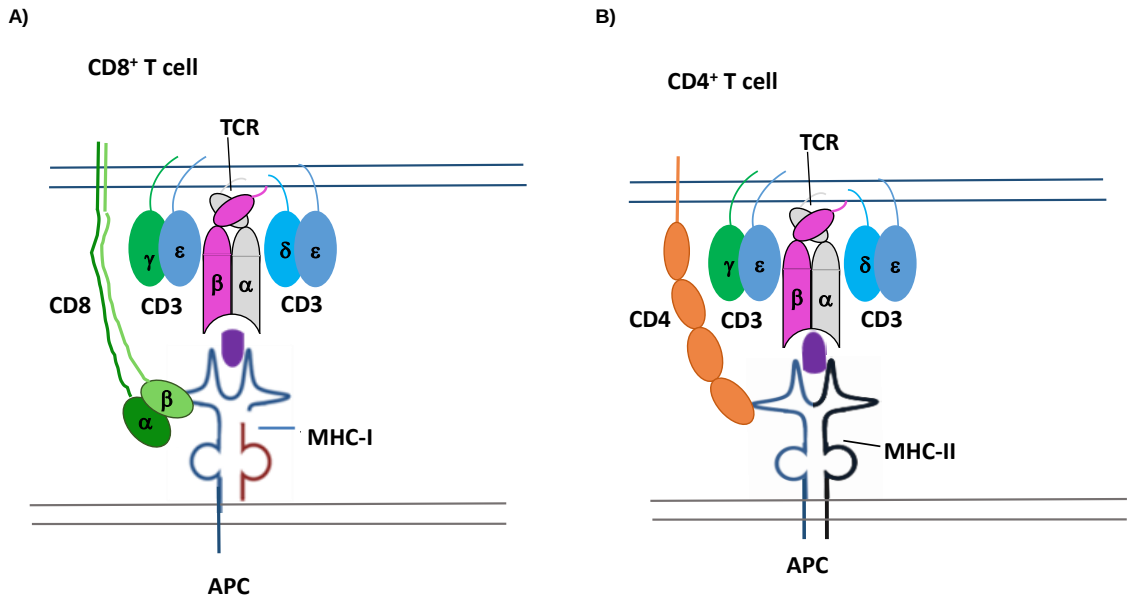

Figure 2. TCR interaction with the MHC complex on $CD8^+$ T cells (MHC-I) (A) and $CD4^+$ T cells (MHC-II) (B).

1.1.1 CD4$^+$ T helper cells

CD4$^+$ T helper cells mainly recognize through the TCR peptides with an average length of 12-15 amino acids derived from extracellular proteins; these are associated with MHC class II molecules. The major role of CD4$^+$ T cells is to coordinate the immune response by direct communication with B lymphocytes and macrophages through immunological synapses, and by the production of cytokines. T helper cells could be divided based on the type of cytokines they released. T helper 1 (Th1) cells secret mainly interleukin (IL)-2, interferon gamma (IFNγ) and TNFα. Th1 cells promote proinflammatory responses, activate macrophages and are highly effective in clearing intracellular pathogens. T helper 2 (Th2) cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines and they promote the antibody responses (7). T helper 17 (Th17) cells produce IL-17, IL-21, IL-6 and TNFα. Th17 cells and their effector cytokines mediate host defensive mechanisms to various infections, especially extracellular bacterial infections, and are involved in the pathogenesis of many autoimmune diseases. The effector cytokines of Th17 cells, therefore, mediate the crucial crosstalk between immune system and tissues, and play indispensable roles in tissue immunity (8). And finally, regulatory T cells (Tregs) that monitor and inhibit the activity of other T cells. They prevent adverse immune activation and maintain tolerance, or the prevention of immune responses against the body's own cells and antigens (9).

1.1.2 Cytotoxic CD8$^+$ T cells

CD8$^+$ T cells are crucial for recognizing and removing virus-infected cells and cancer cells. CTLs have specialized compartments, or granules, containing cytotoxins that cause apoptosis. CD8$^+$ T cells recognize intracellular peptides with an average length of 8-10 amino acids and are associated with MHC class I molecules. Cytotoxic CD8$^+$ T cells carry out their killing function by releasing two types of preformed cytotoxic proteins: granzymes, which seem able to induce apoptosis in any type of target cell, and the pore-forming protein perforin, which punches holes in the target-cell membrane through which the granzymes can enter. Cytotoxic CD8$^+$ T cells also produce cytokines such as IFNγ, which is an inhibitor of viral replication and is an important inducer of MHC class I expression and macrophage activation, TNFα and IL-2.

After activation, the T-cell response is characterised by a stable pool of memory cells that can persist for many years. Then, upon re-exposure to the antigen, memory T cells can respond faster and develop into effector cells more efficiently (10).

1.2 Antigen processing

As it has been previously mentioned, T helper cells and CTLs use their TCRs to recognize peptide antigens presented by molecules encoded by the MHC. The preference for different classes of MHC molecules relates to a demarcation in the antigen-processing pathways that supply peptides. MHC class II molecules generally present peptides derived from exogenous antigens that enter the cell by the endocytic route, whereas MHC class I molecules present endogenously derived antigens, usually synthesized within the cell presenting the antigen. Therefore, CTLs target directly cells infected with virus (11).

The endogenous pathway is used to present intracellular peptide fragments on the cell surface on MHC class I molecules (viral peptides would also be presented, allowing the immune system to recognize and kill the infected cell). Short peptides are generated from the catabolism of endogenous proteins in the cytoplasm through the action of proteasomes and other enzymes, and these peptides arte actively transported into the lumen of the endoplasmic reticulum (ER) by the heterodimeric transporter associated with antigen processing (TAP) where bind to MHC class I molecules. Those peptides will be presented to $CD8^+$ T cells (Figure 3A).

The exogenous pathway is utilized by specialized APC to present peptides derived from proteins that the cell has endocytosed. The peptides are presented on MHC class II molecules. Proteins are endocytosed and degraded by acid-dependent proteases in endosomes where bind to MHC class II molecules. Then, the peptides will be presented to $CD4^+$ T cells (Figure 3B).

Some peptides derived from extracellular proteins can be presented in the context of MHC class I, named cross-presentation. The cell starts off with the exogenous pathways but diverts the antigens to the endogenous pathway. The TAP dependence of such cross-presentation indicates that it involves diversion of the cellular antigens into the conventional MHC class I pathway (11) (Figure 3C).

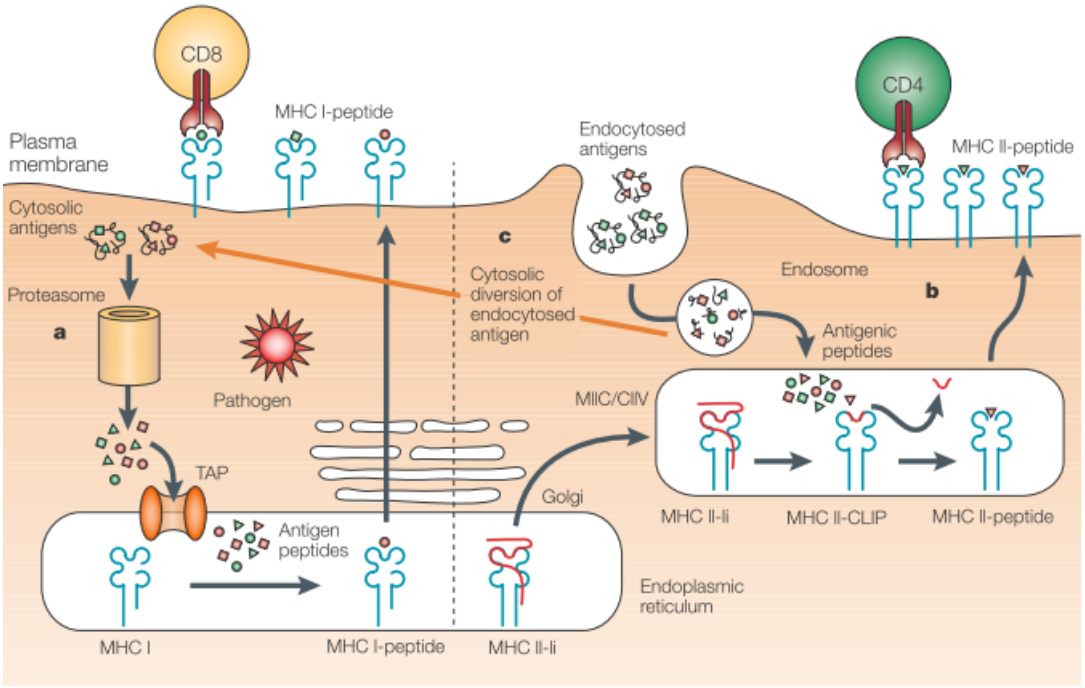

Figure 3. Different antigen-processing pathways for the MHC class I and class II molecules. MHC class I molecules present peptides that are derived from endogenously synthesized proteins of either self or pathogen origin (A). MHC class II molecules present exogenous proteins that enter the cell through the endocytic route (B). Cross-presentation pathway (C). Adapted from Health WR. *et al*. 2001. *Nat Rev Immunol*.

2 Allogeneic haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is a well-established clinical procedure introduced more than half a century ago, consisting of the infusion of stem cells to re-establish haematopoietic function in patients whose bone marrow or immune system is damaged or defective. Over the last 20 years, HSCT practice has increased almost tenfold, becoming the primary indicator for many haematological malignancies and inherited or acquired non-malignant disorders of blood cells (Table 1) (12-14).

In the early 1960s, *allogeneic* HSCT (allo-HSCT) became feasible after the identification of MHC molecules and typing of HLA. The procedure of allo-HSCT requires partial or total elimination of the recipient´s haematopoietic and immune systems through pre-transplant chemotherapy that is sometimes combined with radiotherapy. This conditioning treatment provides space for incoming cells to engraft, helps in preventing graft rejection and kills most residual cancer cells. In this situation, the immune system

plays a crucial role for the transplant success in order to avoid graft failure, graft versus host disease (GvHD) and control of infection.

Table 1. Main malignant and non-malignant haematological disorders of blood cells that are indicated for allogeneic-HSCT.

Malignant disorders	**Non-malignant disorders**
Acute lymphoblastic leukaemia (ALL)	Bone marrow failure syndromes
Acute myeloid leukaemia (AML)	Chronic granulomatous disease
Chronic lymphocytic leukaemia (CLL)	Fanconi anaemia
Myelodysplastic syndrome (MDS)	Metabolic storage disorders
Non-Hodgkin lymphoma (NHL)	Severe aplastic anaemia (SAA)
Hodgkin lymphoma (HL)	Sickle cell anaemia
Multiple myeloma	Thalassemia
High-risk of solid tumours, under certain circumstances	

There are several factors to consider for a successful cell transplant procedure, such as HLA disparity, the disease and its prognosis, time between the diagnosis and the transplant, donor/recipient age and donor/patient viral serostatus.

The HLA system is the major histocompatibility barrier in allo-HSCT as a foreign HLA is recognized by the immune system, and the degree of HLA matching is predictive of the clinical outcome. There are several levels of HLA compatibility between the recipient and the donor that have to be defined prior to performing an allo-HSCT. Based on HLA high-resolution typing for class I (HLA-A, -B, -C) and class II (HLA-DRB1, -DQB1, -DPB1), a well-matched donor is defined as 12/12, 10/10 (when DP is not considered) or 8/8 (when both DQ and DP are not taken into account). DPB1 is only included if several 10/10 matched donors are available. If there is any difference it is considered as a mismatch. In this case, it is possible to have HLA disparity in 1 or 2 loci. There are different types of donors within the allo-HSCT depending on the HLA compatibility:

Related donor

- A sibling that is genotypically identical in both HLA class I and II.
- A sibling or other related familiar donor with HLA disparity in 1 or 2 loci.
- A haploidentical donor that shares one haplotype with the recipient.
- If the sibling is a monozygotic twin, it is a syngeneic transplant.

Unrelated donor

These donors are recorded in the 'Bone Marrow Donor Worldwide' database and the best HLA matched donor will be chosen when possible.

2.1 Stem cell source

There are three main sources of haematopoietic stem cells (HSCs) for clinical transplantation: bone marrow (BM), peripheral blood stem cells (PBSCs) and umbilical cord blood (UCB). BM is obtained by repeated aspiration of the posterior iliac crest while the donor is under general or local anaesthesia. Its use as a source of HSCs has decreased over the years due to the discomfort caused to the donor during the BM isolation. The principal source of HSCs used for both autologous and most allo-HSCTs are PBSCs obtained by an apheresis following patient or donor HSCs mobilization by using granulocyte-colony stimulating factor (G-CSF), which active principle is filgrastim (15). G-CSF causes the proliferation of neutrophils and the release of proteases. Proteases degrade the proteins anchoring the stem cells to the marrow stroma and, together with protease-independent mechanisms, liberate the stem cells to enter the circulation.

The European Group for Blood and Marrow Transplantation (EBMT) survey of over 651 centres about HSCTs activity in Europe during 2011 reported a total of 35,660 HSCT (42% allo-HSCT) and found that over 72% of allo-HSCT were performed using PBSCs (16). Several trials have reported that allogeneic PBSC transplantation can produce a substantially faster engraftment than BM with a reduced rate of relapse, especially in patients with late-onset disease. Moreover, allo-PBSCs improve overall and disease-free survival in patients with more advanced hematologic malignancies. However, in allo-transplantation, PBSCs, which contain more T cells than BM does (despite having similar correlation with incidence of acute graft versus host disease (aGvHD)), increase the incidence and prolong the treatment of chronic graft versus host disease (cGvHD) compared to BM (17). For that reason, BM is a preferable source of HSCs when treating non-malignant diseases.

The use of UCB as source of HSCs has been associated with a decreased incidence of graft versus host disease (GvHD), with the benefits relating to reduced histocompatibility requirements. However, its use has largely been restricted to paediatric and small adults due to the dose limitations, as UCB collections contain a significantly reduced total

number of $CD34^+$ cells, compared with PBSCs. This, can result in delayed or failed engraftment in adults. In the recent years, double UCB transplantations have been carried out to improve survival in adult UCB transplantation, increasing $CD34^+$ cell numbers, therefore reducing the time of engraftment in adult recipients (18).

2.2 Conditioning treatment

Conditioning regimens in allo-HSCT vary in their intensity and can be classified as myeloablative (MA) conditioning and non-myeloablative conditioning or reduced intensity conditioning (RIC) treatments. The purpose of using MA conditioning regimen prior to transplantation is to eradicate the underlying disease and suppress the recipient's immune system to allow engraftment of donor stem cells. The initial MA conditioning treatment was a combination of cyclophosphamide (Cy) and total body irradiation (TBI) (19). In order to avoid possible side effects of TBI, such as pneumonitis, cataracts, endocrinological disturbances, secondary tumours and decreased growth in children, the use of busulfan (Bu) was implemented. Randomized trials comparing Cy-TBI and Bu-Cy in patients with acute myeloid leukaemia (AML) or chronic myeloid leukaemia (CML) found no differences in leukaemia free survival and overall survival between the two conditioning regimens (20).

Due to the high transplant related mortality associated with using the MA conditioning regimen, allo-HSCT was restricted to healthy young patients who are more tolerant to the high dose chemo-radiotherapy regimens. However, the introduction of RIC regimens before allo-HSCT has allowed transplantation in elderly patients and in younger patients with comorbidities (21). RIC regimens minimize toxicities related to MA conditioning treatment but it is necessary profound immunosuppression to allow engraftment of the HSCs, as there is not an eradication of the endogenous bone marrow cells. RIC regimen is based on combination of different drugs such as fludarabine (a purine analogue) combined with melfalan, Bu or Cy.

2.3 Complications following transplant

The high doses of chemotherapy and/or radiotherapy in conditioning regimens affects all organs and tissues of the recipient, producing several early and late secondary effects of variable intensity. The most common early effects are related to gastrointestinal toxicities

as mucositis, esophagitis or gastrointestinal disorders. Other toxicities related to lung, liver and brain have also been described.

However, the main complications that occur after allo-transplantation include engraftment failure, GvHD and infection. In order to avoid the two first complications there are different immunosuppressive prophylaxis treatments available. Thus, both MA and RIC conditioning treatments are combined with cyclosporine-A (Cs-A) or mycophenolate mofetil (MMF) and methotrexate (MTX) to provide post-grafting immunosuppression that impairs T-cell functions (19, 22). Furthermore, when receiving a transplant from an unrelated donor T-cell depletion with alentuzumab or anti-thymocyte globulin (ATG) are also used to avoid GvHD.

a) Failure engraftment can occur when either the haematopoietic process cannot recovering following transplantation or when a recipient's immunocompetent residual cells survive conditioning treatment and refuse donor's stem cells.

b) GvHD is the most frequent complication following allo-HSCT. It can occur despite aggressive immunosuppressive prophylaxis, even when the donor is genetically matched. It is a consequence of interactions between antigen presenting cells of the recipient and mature T-cells of the donor. It can be classified as aGvHD, which normally occurs within the first 100 days post-transplant and cGvHD that normally appears after four months post transplantation. Characteristic tissue damage that normally affects the skin, liver or gut. GvHD is treated with high doses of immunosuppressive drugs (steroids).

c) Infectious complications are one of the major problems that can occur following transplantation. They are due to neutropenia and mucosal and skin damage caused after conditioning and immunosuppression treatments. Most opportunistic infections are caused through viral infections mainly Cytomegalovirus, Herpes simplex, Varicella-zoster and Epstein-Barr (EBV), and bacterial and fungal infectious agents such as Aspergillus and Candida.

3 Cytomegalovirus

Human cytomegalovirus (CMV) is a member of the β-herpesvirus family that infects approximately 70% of the population in developed countries (23, 24). The CMV virion

consists of an icosahedral nucleocapsid (100 nm in diameter) containing a double-stranded linear deoxyribonucleic acid (DNA) genome (230 Kbp). This is surrounded by a proteinaceous layer, defined by a lipid bilayer containing a large number of viral glycoproteins. The mature virion particle is approximately 150-200 nm in diameter (Figure 4).

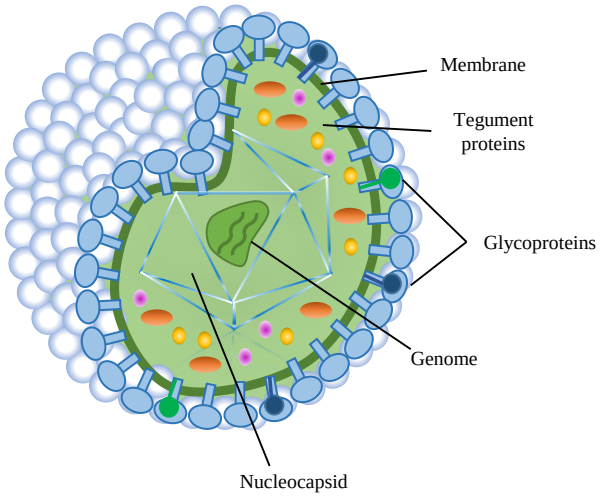

Figure 4. Structure of the cytomegalovirus virion and its components.

Virions gain entry through a membrane fusion event involving the outer membrane of the cell and glycoproteins on the lipid envelope of virions. Once the fusion of these two membranes occurs, the DNA-containing protein capsid and the tegument proteins are released into the cell. During the lytic infection, viral immediate-early genes are expressed which results in the production of viral immediate-early proteins that modulate the host cell environment and stimulate the expression of viral early genes. These proteins are responsible for replicating the double-stranded viral genomic DNA; after DNA replication, these immediate-early genes turn on the expression of viral late genes. CMV-infected cells also produce non-infectious enveloped particles and dense bodies. Non-infectious enveloped particles are defective viral particles composed of enveloped immature capsids that lack DNA, but contain the viral assembly protein (they contain an identical assortment of envelope, tegument and capsid proteins). Dense bodies are enveloped particles that lack an assembled nucleocapsid and viral DNA, but contain several tegument proteins located between the outer lipid membrane and the icosahedral protein capsid. Upon release into the cytoplasm, tegument proteins become functionally

active, where they play important roles in all stages of the viral life cycle, including, viral entry, gene expression, immune evasion, assembly and egress. Several tegument proteins are of particular interest due to their role in the CMV replication cycle, including phosphoprotein 65 (pp65), pp71, pp150 and pp28. These proteins are processed through the proteasome to generate short peptides that would be transported into the ER by TAP to bind to MHC class I molecules and therefore be presented to $CD8^+$ T cells. The pp65 is the most abundant tegument protein and the major constituent of extracellular virus particles and it is delivered to the nucleus of permissive cells at the very start of a lytic infection. Further, pp65 is implicated in countering both innate and adaptive immune responses during CMV infection. Pp65 not only prevents immediate-early proteins from being recognized by components of the immune system, but also inhibits the synthesis of various components involved in the host cell's immune response. The most immunodominant pp65 epitope is NLVPMVATV which has HLA-A*02:01 restriction; however, other CMV-encoded T-cell epitopes with other HLA restriction have also been studied, including major histocompatibility complex (MHC) class II epitopes (25, 26) (Table 2).

Table 2. Most common pp65 epitopes sequences and their HLA restriction.

Pp65 epitopes MHC class I restriction			**Pp65 epitopes MHC class II restriction**		
Residues	**Epitope sequence**	**HLA restriction**	**Residues**	**Epitope sequence**	**HLA restriction**
123-131	IPSINVHHY	HLA-B35	41-55	LLQTGIHVRVSQPSL	HLA-DQ6
188-195	FPTKDVAL	HLA-B35/B68	361-376	PQYSEHPTFTSQYRIQ	HLA-DR11
341-349	QYDPVAALF	HLA-A24	489-503	AGILARNLVPMVATV	HLA-DR3/DR11
417-426	TPRVTGGGAM	HLA-B7	509-523	KYQEFFWDANDIYRI	HLA-DR52
495-503	NLVPMVATV	HLA-A2			
501-509	ATVQGQNLK	HLA-A11			

By looking at the different HLA class I restrictions of CMVpp65 antigen presentation studied, approximately 0.26 (0.18-0.34) of the Caucasian population present HLA-A*02:01 restriction, according to the allele frequencies in the world population website (http://www.allelefrequencies.net/). HLA-A*24:02 restriction is present in 0.10 (0.05-0.21). Around 0.10 (0.01-0.19) of the Caucasian population present HLA-B*07:02 restriction whereas 0.07 (0.03-0.13) have HLA-B*35:01 restriction and 0.06% (0.01-0.13) present HLA-A*11:01 restriction.

The phospholipid envelope contains glycoproteins that play essential roles in viral entry into host cells, cell-to-cell spread and virion maturation. There are two glycoprotein complexes required for virus entry: glycoprotein complex I composed of homodimeric glycoprotein B (gB) molecules and heteroligomeric complex III composed of gH and gL. gB is an essential glycoprotein that plays a crucial role in virus binding, as it is the major cell surface, heparan sulfate proteoglycan-binding glycoprotein. It also participates in viral entry, cell-to-cell spread and cell fusion. On the contrary, gH and gL are necessary for the final stage of virus entry via pH-independent fusion between the viral envelope and the cell membrane. The immunodominant gB epitope is DYSNTHSTRYV (residues: 217-227), whereas HELLVLVKKAQL is the immunodominant gH epitope (residues: 276-287) (27).

3.1 Opportunistic cytomegalovirus infection and host balance

CMV represents one of the most common infections among healthy people and it is a major problem within immunosuppressed patients.

Following primary infection in healthy immunocompetent individuals, the virus and the immune system reach a homeostatic balance, and life-long asymptomatic latency is established. This occurs predominantly in cells of the myeloid lineage, where intermittent sub-clinical reactivations are successfully controlled by the immune system (28). The immunological control of CMV exerted by the host requires a high proportion of the immune repertoire to be directed against this pathogen, with competent CMV-specific $CD4^+$ and $CD8^+$ T cell subpopulations involved (29, 30).

By contrast, CMV infection/reactivation can cause severe disease and even mortality in the absence of an effective immune response, such as immunocompromised individuals and immunological immature of neonates or newborns (31, 32). Recipients of allo-HSCTs are treated with immunosuppressive drugs which target both the $CD8^+$ and $CD4^+$ T-cell compartments, which are critical for CMV immune control. This deficit allows uncontrolled CMV replication and may lead to development of life-threatening end-organ damage. The incidence of CMV reactivation during the post-transplant period is approximately 70–80% in adults and 30–40% in paediatric allo-HSCT (33); primary infection following HSCT occurs in 20-40% of CMV seronegative patients whose donor is CMV seropositive (34). The most common clinical manifestations of CMV disease are

pneumonia and gastrointestinal disease, but retinitis, central nervous system disease and marrow suppression may also be observed (35).

4 CMV immune responses

4.1 Healthy individuals

The time course of the appearance of CMV-specific immune responses in healthy individuals is difficult to follow, as the beginning of primary infection normally goes unnoticed. Once CMV establishes primary infection, several mechanisms and pathways of the innate immune response are activated. Following infection, monocytes, macrophages and dendritic cells release inflammatory cytokines and upregulate co-stimulatory molecules that slow down the pathogen before an adequate adaptive immune response is developed (36). Once the virus disseminates to monocytic cells of myeloid lineage including monocytes and CD34 cells, it establishes latent infection (37, 38). A model of primary CMV infection has been proposed by studying CMV-naïve individuals that received a donor kidney from CMV carriers (24). Based on *in vitro* culture, one week after the peak of CMV replication CMV-specific $CD4^+$ T cells emerge and synthetize Th1 cytokines (IFNγ and TNFα) (39, 40). Following primary CMV infection, CMV-specific $CD4^+$ T cells show a phenotype of recently activated naïve T cells co-expressing CD45RA and CD45RO surface markers, co-stimulatory receptors CD27 and CD28 and the cell cycle-associated nuclear marker ki67 (Figure 5A). Then, CMV-specific $CD8^+$ T cells become detectable in peripheral blood and have an effector memory (T_{EM}) phenotype, characterised by the loss of CD45RA and CCR7 cell surface markers (41). These virus-specific $CD8^+$ T cells express perforin, granzyme B and CD95 and have the capacity of lysing CMV-peptide presenting target cells (42, 43). In the months following primary infection, CMV-specific $CD8^+$ T cells gradually lose CD27 and re-acquire CD45RA expression (known as terminally differentiated effector-memory CD45RA T cell (T_{EMRA})). This seems to increase with age, however the CMV-specific $CD8^+$ T cells still maintain their cytolytic potential (Figure 5B). On the contrary, CMV-specific $CD4^+$ T cells keep an effector memory phenotype which is not related to age (44).

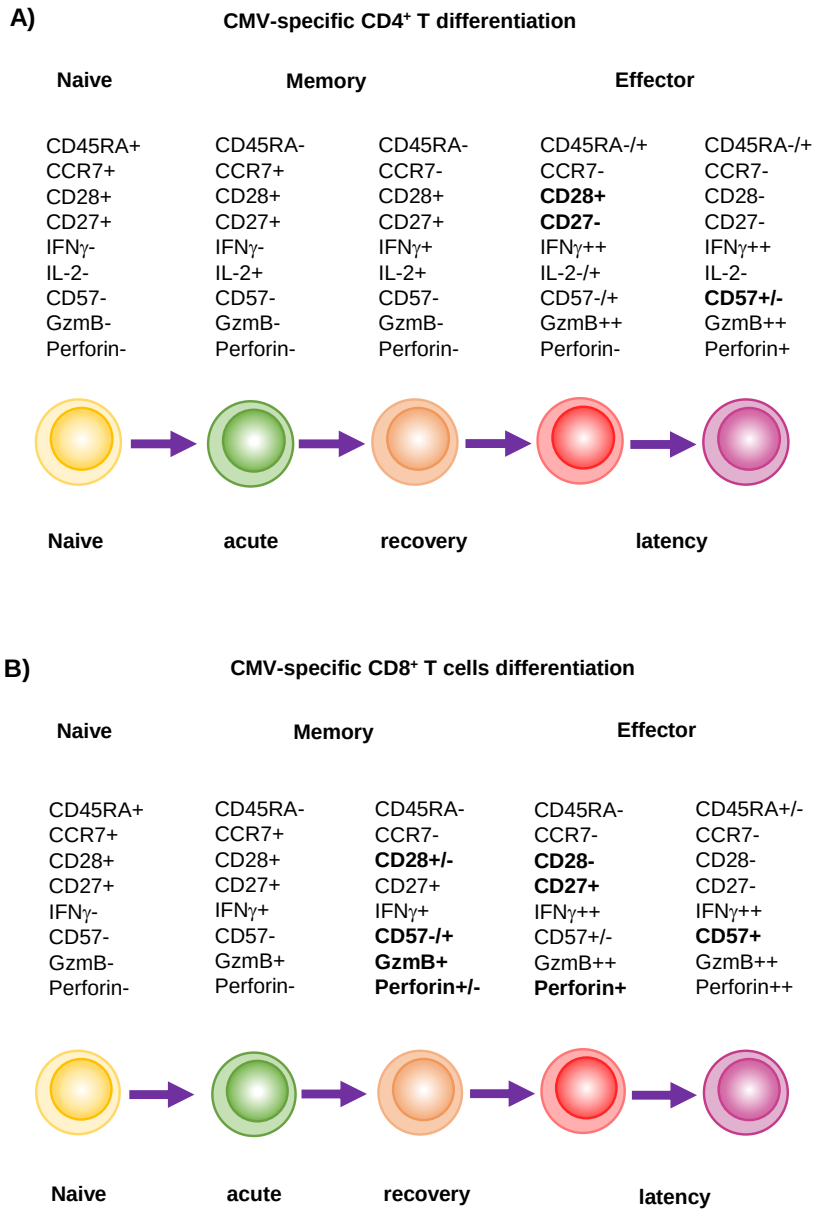

Figure 5. CMV-specific T-cell differentiation. Phenotypic evolution of $CD4^+$ (A) and $CD8^+$ (B) T cells. CMV-experienced T cytotoxic cells exhibit a different functional phenotype compared with naïve cells. Expression of different molecules such as CD45RA, CCR7, CD28, CD27, CD57 or mediators of cytotoxicity, such as IFNγ, granzyme B (GzmB) or perforin.

During latency, CMV-specific cells express the senescence marker CD57 and lose ki67 expression. However, these specific cells are not exhausted and can respond to reactivation of latent virus *in vivo* (45, 46). T-cell exhaustion is characterised by a progressive loss in the ability of $CD8^+$ T cells to produce cytokines (IL-2, TNFα, IFNγ), as well as to survive, proliferate and kill targets. Notably, during chronic CMV infection in healthy individuals, CMV-specific T cells do not upregulate programmed cell death

(PD)-1, an inhibitory molecule strongly associated with antigen-driven T-cell exhaustion (47). Indeed, even in the absence of $CD4^+$ T cells, which accelerates $CD8^+$ T cell exhaustion after chronic CMV infection (48, 49), only a small proportion of $CD8^+$ T-cells are dysfunctional (50, 51). Importantly, the release of Th1 cytokines characterise early and late virus-specific T cells that accumulated during latency. CMV-specific T cells of infected subjects dominate the memory compartments where $CD4^+$ and $CD8^+$ comprise approximately 10% in peripheral blood. However, these T cells, despite restraining viral replication and preventing disease, do not eliminate the virus, which persists as a latent infection in the host (52).

4.2 Immunocompromised individuals

As it was previously mentioned, allo-HSCT recipients are treated with immunosuppressive drugs that target both the $CD4^+$ and $CD8^+$ T-cell compartments. Susceptibility to viral, bacterial or fungal infections in those patients is the result of profoundly reduced innate and adaptive immunity in the immediate post-transplant period caused by the immunoablative effect of the host bone marrow (53, 54). One of the main mechanisms that underlie the immunological tolerance is engraftment of donor HSCs in the host bone marrow. This is followed by the production of donor-derived pro-thymocytes, which undergo maturation in the host thymus and thereby acquire central tolerance to host tissue (Figure 6).

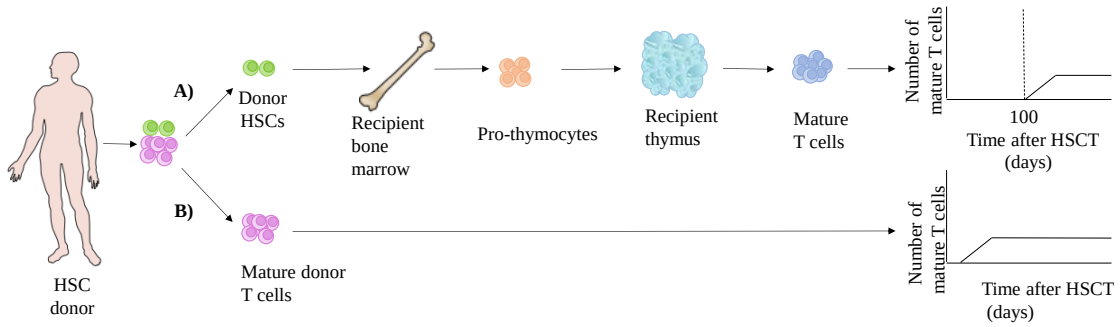

Figure 6. T-cell immune reconstitution after allogeneic haematopoietic stem-cell transplantation. A) Recipient's T-cell recovery is generated after maturation of donor's T-cells from the haematopoietic stem cells (HSCs) after engraftment in host's bone marrow. Donor-derived cells undergo maturation in patient's thymus in the presence of both host and donor tissue. These T cells not only are functionally competent but also fully tolerant of both host and donor tissue. This process takes at least 100 days. B) Mature donor T cells that from the HSC graft can engraft directly in the host, thereby more rapidly providing cellular immunity. However, these T cells are responsible for GvHD and therefore are frequently depleted from the HSC graft during HSC processing or are controlled by the administration of immunosuppressive drugs. Adapted from Moss P.et al. 2005. *Nat Rev Immunol.*

In this microenvironment, restoration of adaptive immunity following allo-HSCT is a slow process. Naïve ($CD45RA^+CCR7^+$) T cells first appear approximately four months after allo-HSCT, and the complete recovery of the naïve T-cell pool may take one to two years. T-cell counts recover much earlier by peripheral expansion (55). Post-thymic donor-derived T cells expand rapidly after allo-HSCT. These cells are central memory ($CD45RA^-CCR7^+$) (T_{CM}) and T_{EM} cells, which are crucial for the success or failure of allo-HSCT due to their impact on the engraftment, GvHD, graft versus leukaemia (donor T-cells that eliminate malignant residual host T-cells) and antiviral therapy (56). In the early stages post-transplant, the mature T-cell repertoire from the donor interacts with the new recipient's environment, leading to clonal expansion against diverse antigens driven by lymphopenia and cytokines. Early clonal expansion of CMV-specific cytotoxic T lymphocytes (CMV-CTLs) depends on the presence of cells with these specificities in the donor (57) and other factors in patient's microenvironment.

Most studies have focused on $CD8^+$ T-cell immunity and have shown that following primary infection there is an increase of CMV-specific $CD8^+$ T cells which is related to a reduction in both CMV reactivation and disease (58-60). Later on during persistent infection, an equilibrium is reached between viral replication and cellular immune responses.

However, several studies suggest that functional CMV-specific $CD8^+$ T cells are not sufficient to control viral replication and that effector-memory $CD4^+$ T cells are necessary for the recovery of infection. It is interesting to mention that whereas in immunocompetent subjects CMV-specific $CD4^+$ T-cell responses precede CMV-specific $CD8^+$ T-cell responses, in allo-HSCT recipients the CMV-specific effector-memory $CD4^+$ T-cell responses are delayed. The CMV-specific $CD4^+$ T-cell response is dominated by large oligoclonal expansions of cells with cytotoxic activity and predominant production of IFNγ (61). The impaired control of viral replication can be explained by the lack of IFNγ secreting effector memory $CD4^+$ T cells at the side of infection in allo-HSCT patients (62). Consequently, functional recovery of specific $CD8^+$ cell-mediated cytotoxicity after transplantation may require expansion and activation of virus-specific T-helper cells (63). In that sense, $CD4^+$ T cells seem to be necessary for the regulation of cell-mediated immunity, promoting cytotoxic T-cell activity through Th1 cytokine elaboration and activation of APCs (64).

Recipients with poor CMV-specific $CD4^+$ and $CD8^+$ T cells responses within the first 100 days post-transplantation have been associated with a high risk of recurrent viral reactivation (65). On the contrary, patients with a positive response against CMV have seemed to harbour lower a virus load and a more rapid clearance of CMV compared to their negative counterparts, suggesting the importance of CMV immunity in clearing the infection in those patients (66).

5 Factors that can module CMV immune reconstitution in allogenic stem cell recipients

CMV reactivation may be influenced by several factors, including donor/recipient CMV serology, degree of HLA disparity, immunosuppression, conditioning regimens and graft manipulation.

5.1 Donor and recipient serostatus

CMV serostatus of the donor has been demonstrated to affect the outcome of the allo-HSCT (67). Therefore, the selection of the donor may depend on the allo-HSCT recipient CMV serostatus. For a CMV-seronegative recipient (R-), it is preferable to use a CMV-seronegative donor (D-), in order to reduce the possibility of primary CMV infection associated with a seropositive allograft (68, 69). On the contrary, stem cells from a seropositive donor (D+) are preferred for a seropositive recipient (R+) (70). CMV-seropositive patients have shown much a higher incidence of CMV infection than CMV-seronegative recipients (71). However, this post-transplant CMV reactivation represents the major factor driving CMV-specific immune reconstitution (72, 73). There are some cases in which immunity was reconstituted in the absence of detected infection, probably due to a silent infection occurring in a target organ. This mechanism may be similar for the immune recovery in seropositive donors and seronegative patients. However, an antigen-dependent, cytokine-driven expansion may help immune reconstitution (74).

Recent studies suggest that D+/R+ transplants generate higher levels of multifunctional CMV-specific T cells, even in the absence of detectable CMV reactivation and also require less antiviral therapy compared with D-/R+, in which CMV-specific cellular immunity reconstitution is dependent on CMV antigen exposure during CMV reactivation (31, 75). This fact may be explained by the transfer of T cells present in the

allograft from a CMV seropositive donor, in which both naïve and memory/effector CMV-CTLs are transferred to the recipient, while grafts from seronegative donors only transfer naïve CMV-specific T cells. Approximately 30% of D+/R- develop primary CMV infection. Despite there being low risk of CMV disease due to pre-emptive treatment of CMV infection, the mortality caused by fungal or bacterial infections is higher in D+/R-compared to D-/R- (18.3% vs. 9.7%, respectively). This is possibly because of the immunosuppressive effects of CMV therapy (76). The kinetics of CMV-CTL reconstitution is different in CMV-seropositive recipients between receiving a transplant from either a CMV-seropositive or a seronegative donor. R+/D- showed a delayed reconstitution of CMV immunity compared to R+/D+ (day +120 *vs.* day +30, respectively) (77). Besides, CMV-CTLs levels are normally higher in the D+/R+ group compared with significantly lower numbers in the other groups (D-/R+, D-/R-, D+/R-) (78).

5.2 Degree of HLA disparity

HLA disparity between the host and the donor is another factor that may contribute to a high risk of CMV infection. As it has been previously mentioned, HLA disparity is associated with aGvHD. The use of steroids drugs to treat GvHD is related to the inhibition of immune function and blockage of T-cell activation. Functional recovery of both CMV-specific $CD4^+$ and $CD8^+$ T cells is impaired due to steroid administration. However, there is not a clear explanation of why HLA disparity may contribute to the risk of CMV infection. According to a study by Borchers et *al.*, GvHD is more frequently observed in patients receiving a transplant from a mismatched donor and therefore, they received steroids treatment that lead to a delay on CMV-specific T-cell immune recovery (78). These observations agree with the results obtained by Mead et *al.* where CMV infection was more frequent in mismatched unrelated donors compared to matched unrelated donors (56% *vs.* 30%) (79). Similarly, the study developed by Jaskula and collaborators showed that a lack of optimal donor/recipient HLA matching was associated with a higher risk of aGvHD and a higher rate of CMV reactivation/infection (68).

5.3 Immunosuppression treatment

The immunosuppressive regimens that allow the recipient to keep the graft and avoid GvHD associated complications also play a role in CMV replication. The state of immunosuppression experienced by the recipient is modulated by factors, such as pharmacologic therapies (type, timing, duration, and sequence), immunogenetic characteristics (HLA match), the presence or absence of immunomodulating viruses, and metabolic abnormalities. Allo-HSCT recipients treated with high-dose corticosteroids (>1 mg/kg/day), MMF and certain anti–T-cell strategies (eg, Campath or ATG) are considered at high risk for CMV disease. In one study, an increased risk of CMV disease or CMV-related complications in allo-HSCT patients was associated with MMF treatment, which seems to upregulate CMV replication (80). Highly immunosuppressed recipients have delayed or reduced immune reconstitution, which has a direct effect on the viral replication dynamics *in vivo* (81). The progression from viral detection to overt disease with a rapidly increasing viral load is occurred in highly immunosuppressed patients (eg, those taking >1 mg/kg/d of corticosteroids) such that any positive test should trigger a requirement for immediate treatment in these patients. Further, viral load may increase in highly immunosuppressed individuals receiving antiviral drugs (82).

5.4 Conditioning regimens

Some studies have investigated CMV immune recovery after allo-HSCT of patients according to pre-allo-HSCT treatment. RIC-allo-HSCT has been associated with lower risk of high-grade of CMV infection; however, this effect does not appear to protect against complications of CMV (83). These observations agree with a study by Kim et *al.* in which they observed that CMV reactivation was less common in RIC-allo-HSCT patients compared to MA, early following allo-HSCT, while there is no difference during the late-recovery period (84). Patients that have received the RIC regimen with alemtuzumab treatment have shown a high rate of early CMV reactivation (85). The numbers of $CD4^+$ and $CD8^+$ T cells remained low within the first three months but they started to recover on day 90. CMV-specific T-cell levels increased 180 days post allo-HSCT. In summary, the higher incidence of CMV reactivation has been related to an inadequate or defective recovery of $CD8^+$ T cells as a consequence of the conditioning regimen (58).

5.5 Graft manipulation

The use of T-cell depletion and G-CSF-mobilized stem cell conditioning protocols are used to improve engraftment and reduce GvHD. However, as these manipulations may be involved in increasing the risk of CMV infection, non-T-cell depleted transplants are preferred when possible.

CD34-positive selection of PBSCs allows GvHD to be minimized by effective reduction of T cells in the graft. A study developed with young patients found that CMV-specific T-cell reconstitution was significantly delayed in patients receiving T-cell depleted grafts by collection of $CD34^+$ progenitor cells, compared to those who received unmanipulated HSCT (median time of CMV-specific T-cell reconstitution of 75 *vs.* 47 days, respectively) (86).

In contrast to CD34-positive selection, CD3/CD19-depleted peripheral allografts contain other immune components, such as NK cells, DCs and monocytes that may be used to generate anti-leukemic, anti-viral or graft-facilitating effects. Recent studies have shown that T-cell recovery after CD3/CD19-depleted grafts achieves normal values within the first 60 days post- transplantation (87, 88). However, when CD3/CD19-depletion is used in combination with RIC regimen, T-cell reconstitution is delayed to 3 months after HSCT (89).

A recently developed method is based on depletion of αβ T lymphocytes couples with B-cell depletion. This approach allows to transfer to the recipient not only high numbers of $CD34^+$ cells and mature donor NK cells, but also γδ T cells which can exert their protective effect against both leukaemia cell regrowth and life-threatening infections. γδ T cells participate in anti-CMV responses, particularly when conventional adaptive immunity is insufficient to clear the viral infection, It has been reported that patients with CMV reactivation after allo-HSCT have a significant expansion of cytotoxic γδ T-cells compared to recipients who did not reactivate (90).

6 Anti-CMV treatment

Prophylactic or pre-emptive antiviral strategies are widely used to control CMV infection following HSCT. The administration of antiviral drugs, which also able to restrain other

herpesviruses, has now become the standard first line therapeutic treatment against primary or reactivated CMV infection and disease. The aim of prophylaxis therapy is to reduce the incidence of CMV infection/disease after transplantation, while the purpose of pre-emptive therapy consists of monitoring the CMV reactivation and early intervention when CMV reactivation is detected.

The first anti-CMV drug used was *immune-globulin*, however its use as a prophylaxis has had no effect in reducing the incidence of CMV disease (69) or CMV infection (91) in allo-HSCT recipients.

Acyclovir and *valacyclovir* have been used as prophylactic treatment to treat herpes-simplex virus with a reduction in the risk of CMV infection/disease, improving survival within 100 days post- transplantation. However, it should be combined with a pre-emptive strategy by using sensitive assays in order to monitor CMV reactivation (92).

Ganciclovir (GCV) has been used not only as prophylactic therapy for preventing early CMV disease after transplantation, but also as pre-emptive therapy in patients with CMV viremia. Although GCV prophylaxis is considered an effective strategy for preventing CMV disease early after transplantation, it is associated with drug-related toxicities (myelosuppressive and immunosuppressive effects) (93) and it has been found to impair and delay the development of CMV-CTLs (94, 95). The use of GCV at the onset of CMV viremia and discontinuing treatment once follow up monitoring assays become negative has been shown to provide adequate CMV disease prevention with less toxicity than prophylactic treatment (96-98). However, it is associated with delayed CMV immune recovery. Patients treated pre-emptively developed protection against CMV at a median time of 139 days compared to those that resolved CMV infection or did not have infection (median of 70 days) (86).

Valganciclovir (VGC), which is an oral pro-drug of GCV, has also been found to be an adequate alternative to intravenous GCV for the prevention of CMV disease in haematopoietic transplant patients as its bioavailability is 10-fold higher (99); however its use is limited by neutropenia (100).

An alternative to GCV is *foscarnet*, which is as effective as GCV (101). GCV and foscarnet have different toxicity profiles; GCV is associated with myelotoxicity whereas foscarnet produces nephrotoxicity. It has been shown that foscarnet causes less

neutropenia than GCV. Therefore, it should be used in the pre-emptive therapy against CMV infection for patient with neutropenia or developing neutropenia during therapy with GCV, however it also delays CMV immune reconstitution (86). *Cidofovir* is a new antiviral drug that can be used when patients fail the first-line of antiviral treatment (35, 102).

Newly available drugs are currently being studied for their potential as prophylactic treatment, such as *maribavir* which causes inhibition of viral encapsidation. *In vitro* studies have shown that maribavir is more potent than GCV against CMV. In a phase II dose-ranging study, it has been found that maribavir was safe and well tolerated and effectively reduced CMV infection after allo-HSCT, compared to placebo (103). However, it was also associated with an increase of adverse events, such as an increased incidence of taste disturbance, nausea and vomiting, but laboratory adverse effects were not more common than in placebo recipients. By contrast, in a phase III study (104), maribavir prophylaxis did not show superiority to placebo in prevention of CMV disease when started after engraftment. Instead, it only showed a modest antiviral effect in prevention of CMV reactivation. There were some explanations provided for the failure of the trial and further trials might be necessary to sufficiently test the potential of this antiviral drug.

Brincidofovir (CMX001) is another available antiviral agent that has shown potent *in vitro* activity against CMV (105). It is converted intracellularly to cidofovir. It is not concentrated in renal proximal tubules and is unlikely to have renal toxicity. It has been shown to reduce the incidence of CMV events, as compared with placebo and no evidences of increased myelosuppression or nephrotoxicity were found.

Letermovir is another antiviral drug that has been studied (106). Oral letermovir is currently in a phase II trial and seems to be a highly active anti-CMV agent with a novel mechanism of action compared to GVV, VGC, foscarnet and cidofovir. It provides a potential new treatment option for patients infected with CMV strains that are resistant to approved previous antiviral drugs. It reduces the incidence of CMV infection in allo-HSCT, when comparing with the placebo and the incidence of neutropenia was similar to placebo (7% *vs.* 6%), which is quite different from neutropenia observed with VGC (58%). Further to this, it was found to be as safe as placebo, with no apparent safety concerns, such as haematological and renal toxicity.

Despite the need of prophylactic or pre-emptive treatment for CMV infection early after transplantation, life threatening complications are related to the, such as secondary bacterial and fungal infections (107, 108). The incidence of CMV disease within the first 4 months in patients following allo-HSCT has been reduced due to the use of prophylactic and pre-emptive strategies to less than 5% (33, 109). These treatments, however, can lead to myelosuppression, thrombocytopenia and other drug related toxicities such as renal and metabolic damage (35). However, approximately between 10%-30% of patients develop a delayed onset of CMV disease after 100 days post-transplant as a result of antiviral prophylaxis and early intervention (110, 111). This may be due to the resistance to the drugs, as they share similar mechanisms of action (112), or due to delayed reconstitution of CMV-specific T-cell responses, representing one of the leading causes of mortality after allo-HSCT (94, 113, 114). For all these reasons, many trials have assessed the effects of the combination of antiviral drugs with therapies based on infusion of virus-specific primed T cells (115).

6.1 Viral-specific adoptive immunotherapy after allogeneic HSCT

It has previously been demonstrated that there is correlation between levels of CMV-CTL responses and improved control of CMV viremia in immunocompromised HSCT patients. Looking at these observations, many groups have focused their interest on developing strategies for adoptive transfer of CMV-specific T cells. Previous studies have shown that infusion of donor-derived CMV-specific $CD8^+$ T-cell clones or cell lines can successfully transfer protective immunity (116-118), and numerous *in vitro* studies have defined the best methodology for the expansion and selection of virus-specific T lymphocytes for clinical use. This includes: 1) classic *ex vivo* expansion, where T cells are stimulated with APCs that have been transduced with either a viral vector or plasmids encoding the antigens of interest; 2) multimer selection of the specific T cells with magnetic beads; 3) capture of T cells that secret cytokines after stimulation with viral antigens, allowing antigen-specific T-cell isolation by magnetic selection (119-123); methods based on genetic engineering that redirect the specificity of T cells, in order to make them recognize the antigen of interest, by the introduction of TCR genes or chimeric antigen receptors (124). Studies by Feuchtinger et *al.* and Einsele et *al.* have indicated the significance of antiviral effector functions of T-helper cells in maintaining CTL responses after adoptive transfer (117, 125). Few studies in phase I/II have demonstrated the

feasibility of transferring CMV-specific T cells whereby CMV-specific immune restoration can be accelerated without serious immediate infusion-related toxicities. However, some patients can develop GvHD after infusion, which is correlated with clinical protection (117, 118, 126, 127).

These promising results confirm that cellular immunotherapy can accelerate recovery of antiviral immunity in allogeneic HSCT recipients, with important clinical benefits, such as reduction of secondary viral infection episodes (128, 129).

6.2 Mesenchymal stem cells for treatment of CMV infection

One of the primary complications in patients following allo-HSCT is the development of GvHD. Due to the lack of efficiency of existing methods for GvHD prophylaxis, new methods are being actively explored, including the use of donors' multipotent mesenchymal stem cells (MSCs). These cells are fibroblast that can differentiate into osteoblasts, chondrocytes, adipocytes and myoblasts. MSCs exhibit extensive immunomodulatory activities and they affect a broad panel of immune cells of the innate and adaptive immunity. It has been demonstrated that upon stimulation with inflammatory cytokines, MSCs exhibit broad-spectrum antimicrobial effector functions directed against a range of clinically relevant bacteria, protozoal parasites and viruses; therefore, exhibiting a potent antimicrobial effector function (130). However, a later study has shown that CMV-infected MSCs lose their cytokine-induced immunosuppressive capacity and are no longer able to restrict microbial growth (131). Nevertheless, some researchers remain interested in the use of MSCs as treatment for viral infections and it is currently being evaluated the efficacy of MSCs is currently being evaluated in the treatment of refractory CMV infection after allo-HSCT in a clinical trial (https://clinicaltrials.gov/ct2/show/NCT02083731).

7 Detection methods for CMV presence and virus-specific T-cell responses

Detection of early CMV replication is essential in order to initiate CMV antiviral treatment before CMV disease and related-complications occur. During the past years, several assays have been developed to detect the presence of CMV in blood and to measure antigen-specific T-cell responses, which can be compared, depending on their applicability in research and clinical monitoring.

7.1 CMV antigenemia assay

A CMV antigenemia assay has been commonly used for more than a decade for quantification of CMV in blood specimens (132, 133). Detection of the viral pp65 antigen, a structural protein expressed in blood leukocytes during the early phase of the CMV replication cycle, has been performed in allo-HSCT recipients to guide pre-emptive therapy of CMV reactivation, preventing its progression to disease (134). The test result gives a quantitative measurement with a strong correlation between viremia and clinical disease severity. Nevertheless, looking at its use in the clinical practice it has several limitationss: samples should be processed within 6-8 hours of blood extraction, the result is influenced by leukocyte number and operator subjectivity. The lack of evidence to define breakpoints of the test and conflicting evidence on its sensitivity and specificity in detecting CMV disease provide the need in the search for newer testing methods that may be more reliable for clinical monitoring.

7.2 Polymerase chain reaction

The CMV specific polymerase chain reaction (PCR) has been established as a way to detect the presence of CMV DNA and to reduce the duration and side effects of antiviral therapy-based on patients following allo-HSCT (135). However, qualitative PCR may not discriminate latent CMV infection from replication CMV infection. The sensitivity of this technique has been shown to be 100% but the specificity for CMV disease was less than 50% (136). Therefore new advances in this technique were developed in order to detect virus replication.

Quantitative PCR (qPCR) is used to simultaneously amplify and quantify a targeted DNA molecule. The general principle of PCR is maintained but with the feature that the amplified DNA is detected as the reaction progresses in real time (137). This method is an important monitoring tool, being of increased sensitivity for detection of early viral replication, even during severe neutropenia. This method has been widely used for the monitoring of CMV reactivation in allo-HSCT patients in the early post-transplant period. It enable processing a great number of samples with high sensitivity in a short period of time. However, occasional unspecific reactions have led to false positive results and there is currently no clear agreement on the ideal cut-off for the diagnosis of active CMV infection.

Several authors have compared this method with the CMV antigenemia assay suggesting that qPCR could be more useful due to its effectiveness in monitoring CMV disease progression and in guiding therapy (138). Besides, qPCR could be more valuable for an early diagnose of CMV-gastrointestinal disease compared to the antigenemia assay (139). This method could therefore be useful not only for monitoring viral reactivation but also for establishing new preventive and therapeutic strategies against the virus (140).

7.3 Intracellular cytokine staining

Intracellular cytokine staining (ICS) is a flow cytometry-based method, which allows the determination of both the phenotype and cytokine-production, after stimulation with an antigen of interest, of individual cells (141, 142). This method allows the measurement of a broad spectrum of different phenotypic markers and cytokines at the same time. $CD8^+$ or $CD4^+$ antigen-specific T cells can be detected when using the respective antigens in a single assay (HLA-class I or HLA-class II restricted peptides, peptide pools or proteins) (143, 144). The detection limit of ICS is in the region of 0.1% or slightly lower, however, populations lower than 0.01% cannot be analysed as they would be within the background generated by spontaneous cytokine-secreting cells (145-147). This assay is commonly used by researchers to study CMV-specific immune recovery after allo-HSCT. Cells are stimulated with pp65 protein, CMV lysate or pp65/immediate early (IE)-1 peptides, and the production of IFNγ or TNFα by CMV-specific $CD8^+$ or $CD4^+$ T cells is quantified.

7.4 ELISPOT

The enzyme-linked immunosorbent spot (ELISpot) assay allows the detection of individual cells secreting a particular cytokine. Cells are stimulated with the antigens of interest to detect antigen-specific cells in wells coated with anti-cytokine antibodies. The secreted cytokines can then be detected by using an enzymatically labelled agent and an insoluble chromogenic substrate reaction produces a spot at the side of each cytokine secreting cell (143, 148). The ELISpot analysis is the *ex vivo* assay with the lowest limit detection to identify cytokine-producing T-cells (146, 149). Moreover, this technique requires a 10-fold less blood sample volume than flow cytometry analysis and has the lowest background compared with other methods of measuring virus-specific cells, such as multimer or ICS. ELISpot assay has been used to monitor the reconstitution of CMV-

specific T cells in allogeneic transplanted patients' blood (150). It can be used to simultaneously screen a much higher number of samples with antigens of interest compared to other methods, as it is performed on 96-well plates (151). However, this method has some disadvantages. As it is not possible to determine the phenotype or the intra-cytoplasmic markers of the cytokine secreting cells, the specific cells cannot be isolated based on their physical characteristics. It also has long time-consuming protocols (i.e. 4 days) (94, 150, 152). Furthermore, ELISpots may underestimate the magnitude of the T-cell response (145, 148). There is also a certain degree of subjectivity in the interpretation of the results, as a threshold for the size, intensity and gradient of spots are user-defined. Several authors have used this methodology to monitor CMV-immune responses by measuring IFNγ production. In that sense, studies based on ELISpot and ICS have shown that CMV responses were only detectable at day 45 with ELISpot, compared to ICS (median response in patients with viremia of 82 spot-forming cells compared to 0% of both $CD4^+$ and $CD8^+$ $CD69^+IFN^+$) (153). Sukdolak and colleagues have shown that the detection of memory T cells with IFNγ-ELISpot assay correlates with the detection of IFNγ after stimulation with CMV pp65 epitopes (154).

7.5 Multimer technology

The development of multimer technology has been largely developed to monitor and purify T cells with a known antigenic specificity. The basis for this technology resides in the recognition of antigen-specific TCRs by a recombinant class I or class II molecule complex bound to a certain immunodominant peptide. Identification of antigen-specific CTLs regardless of their biological activity allows the preparation of a heterogeneous T-cell population. This avoids previous phenotypic characterisations required for the identification of primed subpopulations with long-term survival capacities. Consequently, this staining technology allows the isolation of T cells with a given antigen specificity from seropositive donors without any further manipulation (128). This technique enables the visualization, enumeration, phenotypic characterisation and isolation of virus-specific CTLs from *ex vivo* samples. This technology enables to assess the dynamics of immune response to viral infections, allowing us to determine the frequencies of antigen-specific T cells. Multimer staining can be easily implemented in clinical routine of monitoring patients for immune reconstitution and the results are available within 2-3 hours. Besides, multimers enable the characterisation of T-cell

phenotype in *ex vivo* samples without requiring the alteration of phenotype by antigenic stimulation. It is possible to identify T cells that keep the T-cell receptor (TCR) but do not maintain their functional activity.

Nowadays, there is a wide variety of available MHC multimer molecules, such as dimers, tetramers, pentamers (PMs), streptamers, dextramers and octamers (155). The most common format in use are MHC class-I tetramers, which are recombinant molecules formed by 4 MHC subunits that have been biotinylated. They are folded with the peptide of interest and tetramerized by a fluorescence-labelled streptavidin molecule (156). Tetramers (Figure 7A) are commonly used by researchers when studying the immune response to CMV in allo-HSCT recipients (77, 157, 158). However, the tetrahedral disposition of MHC molecules in the complex enables it to bind to just three TCR molecules at once, in comparison to PMs, which can bind to five TCRs at a time. PM complexes (Figure 7B) complexes contain five MHC class I subunits that are multimerized by a self-assembling coiled coil domain. All five MHC-peptide molecules face in the same direction resulting in a very high avidity interaction with the TCR. It also comprises up to five fluorescent or biotin tags for bright and efficient labelling compared to tetramer staining (159). PM has also been used to monitor CMV responses (160). More recently, reversible multimers, such as streptamers or histamers have been developed, allowing the dissociation of the multimer from the antigen-specific cell by adding a competitor molecule (161, 162). To constitute the streptamer (ST) complex (ST) (Figure 7C), Strep-tags are fused to MHC-molecules forming MHC-I-Strep fusion proteins, which allow MHC oligomerization by joining to the Strep-Tactin structure that can be either fluorescently or magnetically labelled. These can be used for efficiently stain or isolate antigen-specific T cells (MHC Streptamer Manual, IBA, www.iba-lifesciences.com). The addition of the competitor molecule D-biotin allows the monomerization of the MHC molecules that are spontaneously released from cell surface. These ST-selected specific-CTLs can either be expanded or cloned and then be adoptively transferred to the patient (163), offering a new therapeutic approach at good manufacturing practice. STs have been extensively used to identify and select CMV, EBV and Adenovirus-specific T cells from healthy donors. Their transfer to immunocompromised hosts has shown excellent results, and they are being tested in various clinical trials.

New advances in flow cytometry have shown the possibility of parallel detection of a multitude of different T-cell populations in a single sample by using a multidimensional encoding of MHC multimers (164, 165) enhancing the sensitivity of detection.

However, while multimers are powerful research tools, they also have some disadvantages as multimer staining is HLA-specific and peptide-specific. Furthermore, it is not possible to assess the functional status of antigen-specific T cells. It is therefore necessary to combine multimer staining with other techniques, such as ICS to assess whether the virus-specific T cells detected are functional or not. The main limitation of multimer staining is MHC polymorphism requiring a multimer for each HLA allele would be necessary. In addition, for each allele, specific restricted epitopes have to be identified. Therefore, this technique is limited by available HLA alleles and known epitopes (143). Whilst there are various MHC class I molecules available, MHC class II multimers are more difficult to obtain presumably due to structural differences between class I and II molecules and low avidity of binding between the TCR and MHC class II-peptide complexes. Unfortunately, there is no good class II multimer available for CMV detection (152).

All of these assays detect specific-T cells without *in vitro* expansion; but each assay has several advantages and disadvantages. As a summary, multimer analysis can detect peptide-specific T cells and characterise them phenotypically, however, it is limited to certain HLA alleles and does not provide functional activity information of the detected T cells. It is possible to analyse antigen-specific cytokine expression by using enzyme-linked-immunosorbent-based assay (ELISA), ELISpot and qPCR (IFNγ, TNFα, granzyme B), but the phenotype cannot be analysed at the same time. Besides, the time of performance of each assay varies between few hours (multimer) to several days (ELISpot). Taken these points together, it is not possible to definitively recommend one assay over the others due to the benefits each assay offers. Nevertheless, a combination of some of them could give a better idea of the behaviour of these cells.

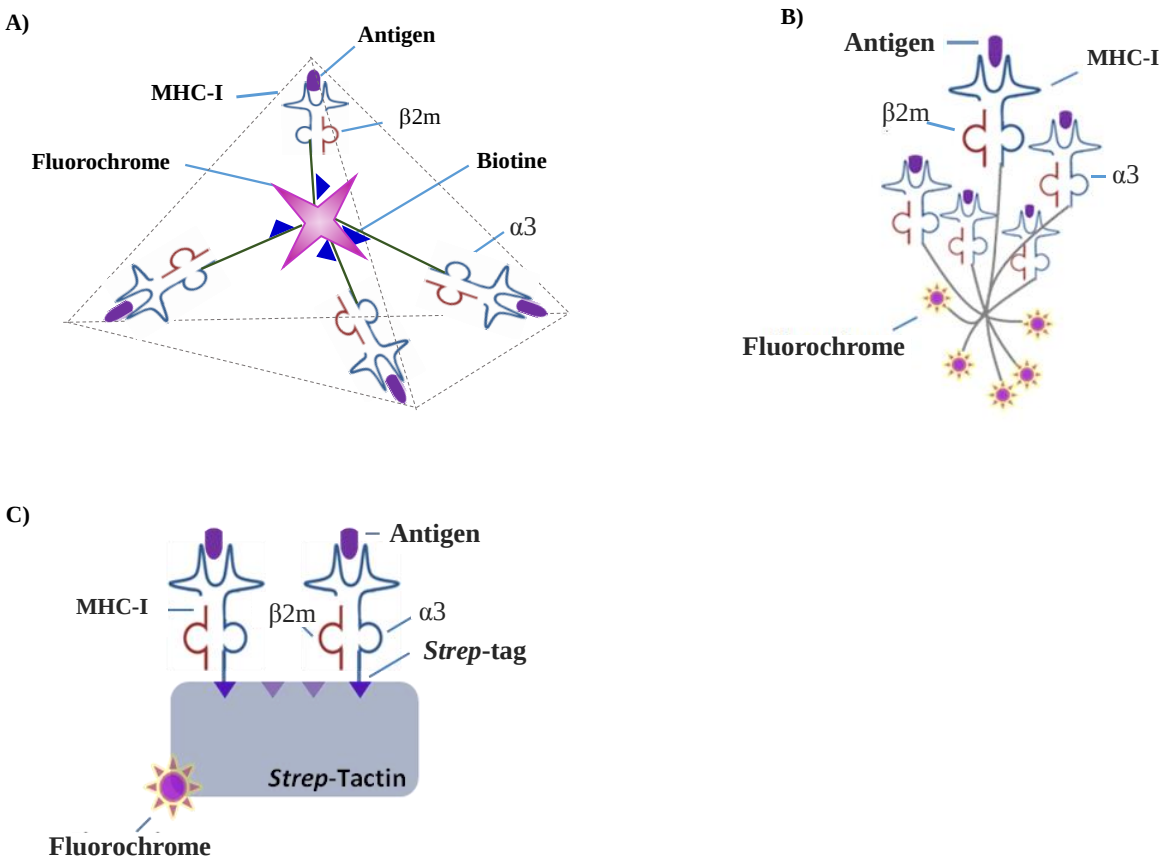

Figure 7. Structure of MHC class I multimers. A) Tetramer complex consists of four MHC class I molecules that are biotynilated and bind to streptavidin fluorochrome-labelled. B) Pentamer complex contains five MHC class I molecules facing the same direction that are multimerized by a self-assembling coiled-coil domain. C) Streptamer complex consists of MHC molecules that are bound to Strep-Tactin by Strep-tag.

7.5.1 Improving MHC class II detection

The majority of the CMV-specific $CD4^+$ T cell monitoring after transplantation is based on cytokine production following stimulation with viral lysates or peptides, or the use of T-cell clones (71). This is due to the lack of reagents, allowing analysis directly *ex vivo* at a single cell level.

The development of class II multimers to characterise $CD4^+$ T cells have shown much slower progress and have been less successful than MHC class-I multimers (166). One limiting factor is that as the α- and β-chain complex is formed irrespective of the antigenic peptide, the proportion of folded class II/peptide complexes in the preparation can be highly variable for different peptides. Another limiting factor is the binding affinity of antigenic peptides derived from tumour and self-antigens, which is generally lower than

that of peptides from pathogens (167). In peripheral blood, frequencies of antigen-specific $CD4^+$ T cells are lower compared to $CD8^+$ T cells: 10^{-3} to 10^{-4} for boosted cells, 10^{-4} to 10^{-5} for resting memory cells and 10^{-5} to 10^{-7} for naïve cells (168, 169). Another limiting factor is the insensitivity of detection in *ex vivo* samples and paucity of defined $CD4^+$ T-cell epitopes with known HLA restriction. In the last years, more epitopes have been defined, so the utility of HLA class II tetramers is expanding (170). CMV-MHC class II tetramers have been previously studied without successful results, suggesting that it is necessary an enrichment step using magnetic beads to allow accurate definition of a responder population (171). Fortunately, the development of MHC class II multimer complexes is being encouraged by biotechnology companies (172) and some studies are now incorporating them for identification of CMV-specific $CD4^+$ T cells upon *in vitro* stimulation (28, 173).

II) HYPOTHESIS AND AIMS

Hypothesis

Immunological monitoring of CMV-specific $CD8^+$ T cells, in conjunction with virology monitoring after transplantation may allow prediction of CMV reactivation after allo-HSCT and assess the risk of CMV-related complications. The characterisation of CMV-specific $CD4^+$ T cells by tetramer technology in healthy donors could help to identify their role in CMV reactivation control prior to its implementation in the monitoring of CMV following allo-HSCT.

These are the aims of this thesis:

1. To optimize new multimer approaches of detection of CMV-specific $CD8^+$ T cells in order to select the best strategy for the incorporation into clinical monitoring practice.

2. To correlate CMV-specific $CD8^+$ T-cell levels and their functionality with CMV reactivation characteristics in allo-HSCT recipients.

3. To characterise the phenotype and functionality of CMV-specific $CD4^+$ T cells by direct staining with tetramer multimer *ex vivo* in healthy individuals prior to introducing it in CMV immune monitoring in allo-HSCT recipients.

III) MATERIAL AND METHODS

1 Healthy donors

Samples from 34 healthy volunteers were used to compare two multimer staining techniques for the monitoring of CMV-specific $CD8^+$ T cells. Repeatability, sensitivity and specificity was assessed for each technique before application in patient samples. Their characteristics are shown in Table 3.

Table 3. HLA and CMV serology of healthy donors.

Donor	HLA restriction	IgG CMV serology
1	A*32, A*36	positive
2	A*24, A*33	positive
3	A*26, A*31	positive
4	A*01, A*03	positive
5	A*29, A*68	positive
6	A*03, A*24	negative
7	A*01, A*24	negative
8	A*29, A*29	negative
9	A*03, A*30	negative
10	A*11, A*23	negative
11	A*02:01, -	negative
12	A*02:01, A*24	negative
13	A*02:01, A*24	negative
14	A*02:01, A*03	negative
15	A*02:01, -	negative
16	A*02:01, A*29	positive
17	A*02:01, -	positive
18	A*02:01, A*01	positive
19	A*02:01, A*24	positive
20	A*02:01, A*30	positive
21	A*02:01, A*24	positive
22	A*02:01, A*24	positive
23	A*02:01, A*33	positive
24	A*02:01	positive
25	A*02:01	positive
26	A*02:01	positive
27	A*02:01	positive
28	A*02:01	positive
29	A*02:01	positive
30	A*02:01	positive
31	A*02:01	positive
32	A*02:01	positive
33	A*02:01	positive
34	A*02:01	positive

2 Patients

This research was approved by the Navarra Government Institutional Review Board. A total of 25 HLA-A*02:01 patients (median age, 42, range 24-65) who underwent allo-HSCT between May 2010 and September 2014 were studied for CMV-specific T-cell reconstitution after written informed consent was obtained. We have performed the study in HLA-A*02:01-restricted recipients because approximately 30% of the Spanish population present this HLA restriction (by looking at http://www.allelefrequencies.net/).

Patients were recruited from the Haematology Service from the Complejo Hospitalario of Navarra (CHN). All donor-recipient pairs were typed at the allelic level for HLA class I and class II loci on DNA samples by reverse PCR-sequence specific oligonucleotide (SSO) methodology (LifeMatch assay; Gen-Probe, Inc., Stamford, CT) and for high-resolution by direct sequencing (AlleleSEQR assay; Abbot Labs, Des Plaines, IL) at the Immunology Unit from CHN. Before transplantation, serological screening of IgG and IgM for CMV was developed by using a chemiluminescent microparticle immunoassay in the Architect system (Abbot Laboratories) at the Microbiology Service of CHN. Values of more than 6 arbitrary units (AU)/mL were considered as positive for CMV serology. Routine PCRs were also performed by using qPCR serum samples from recipients by using a thermocycler (Smartcycler, Cepheid). Demographic and clinical characteristics of each patients are list in Table 4.

2.1 Monitoring of CMV viral load, pre-emptive therapy and schedule of T-cell monitoring

Monitoring of CMV viral load in whole blood was performed by qPCR (Cepheid, Izasa, Scientific) methodology. From the day of transplant viral load was assessed by PCR on a weekly basis from months 1-3 post-transplant, on a biweekly basis months 4-6 post-transplant. Thereafter, patient CMV viral load is assessed on a monthly basis. In addition, PCR was performed weekly during reactivation, or during continuous high immunosuppressive therapy when GvHD was developed. No patient received CMV prophylaxis. Pre-emptive therapy was initiated when viral loads above 500 copies/mL were detected. Patients were treated with intravenous (i.v.) ganciclovir (5 mg/kg/12h) or oral (p.o.) valganciclovir (900 mg/12h) for 2 weeks. Maintenance dose of i.v. ganciclovir (5 mg/kg/day) or p.o. valganciclovir (900 mg/day) for additional 1-2 weeks was

administered. Foscarnet (i.v. 90 mg/kg/12h) was added to the therapy schedule or used to replace ganciclovir or valganciclovir after initiation of pre-emptive therapy in case of neutropenia (<1.0 x 10^9/L) or increasing DNA-viremia (DNAemia) despite therapy. Pre-emptive therapy was discontinued after negative PCR result or a clinically significant decrease in viral load. Cidofovir (i.v. 3-5 mg/kg/week) was administered to three patients to treat either CMV disease or CMV resistance to antiviral drugs.

Table 4. General characteristics of each patient analysed.

Patient	Sex	Age	Type of HSCT	Donor/patient CMV serology	Conditioning treatment
1	F	43	SIB	pos/pos	RIC
2	F	41	URD	neg/pos	MAT
3	F	33	URD	neg/pos	RIC
4	M	55	SIB	pos/pos	MAT
5	F	32	SIB	pos/pos	MAT
6	F	64	SIB	pos/pos	RIC
7	M	58	URD	neg/pos	RIC
8	M	57	URD	pos/pos	MAT
9	F	39	URD	pos/pos	MAT
10	M	27	SIB	neg/pos	MAT
11	M	44	URD	neg/pos	MAT
12	F	65	SIB	pos/pos	RIC
13	M	65	SIB	pos/pos	RIC
14	F	30	SIB	pos/neg	RIC
15	F	52	URD	neg/pos	MAT
16	M	39	URD	pos/pos	MAT
17	F	24	haplo	pos/pos	RIC
18	M	34	URD	pos/pos	RIC
19	M	39	haplo	pos/pos	RIC
20	M	37	URD	pos/pos	MAT
21	M	53	SIB	neg/pos	RIC
22	F	28	URD	pos/pos	RIC
23	M	47	SIB	pos/pos	RIC
24	F	64	URD	pos/pos	RIC
25	M	42	URD	neg/pos	MAT

M = male; F = female; SIB = sibling; URD = unrelated donor; haplo = haploidentical donor; pos = positive; neg = negative; RIC = reduced intensity conditioning; MAT = myeloablative treatment.

Immunologic monitoring was performed every 15 days post-transplant until day +90, monthly until day +200 and every two months to complete the year follow up. A total of 330 blood samples from the 25 patients (median 13 samples; range 4-24 samples) were

analysed. One patient withdrew from the study after two months post-transplant and 5 patients died before the 1 year follow up.

2.2 Management of patients for HSCT

All patients but one received progenitor cells following mobilization from donor peripheral blood, and only one patient received donor BM cells. MA conditioning regimen was performed in 11 patients and 14 patients received RIC regimen. GvHD prophylaxis consisted of Cs-A associated with MTX for patients receiving and HLA-identical sibling allograft. Patients transplanted from an unrelated donor were given alemtuzumab on days -10 to -5 before transplantation in addition to Cs-A and short-term MTX. Acute GvHD, developed in 11 patients, was initially treated with steroids, whereas patients with steroid-resistant disease received MMF (n=3) and mesenchymal stromal cells (n=3).

3 Blood sampling

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque^{TM} separation solution (GE Healthcare Bio-Sciences, Uppsala, Sweden) density gradient centrifugation from ethylendiaminetetraacetic acid (EDTA) anticoagulated blood samples. A total of 15 mL of blood was extracted at each time point from patients. The viability of PBMCs was always >95%, as determined by Trypan blue staining (0.4% Trypan Blue Solution; Gibco, Carlsbad, CA) using a Neubauer chamber (Hausser Scientific, Horsham, PA).

A Detection of CMV-specific CD8$^+$ T cells

1 CMV-specific CD8$^+$ T-cell detection by multimer staining

Two available strategies of multimerization directed against the epitope NLVPMVATV (495-503) of the CMV pp65 protein in the context of HLA-A*02:01 were used:

Pentamer

Pentamer (PM) complex (Figure 3B), HLA-A*02:01/CMVpp65$_{495\text{-}503}$ PM*phycoerythrin (PE) that was synthesized at Proimmune (Oxford, UK).

Streptamer

Streptamer (ST) molecule (Figure 3C), HLA-A*02:01/CMVpp65$_{495\text{-}503}$ ST*PE which was synthesized at IBA (Gottingen GmbH, Germany). To constitute the ST complex, 0.75 µg PE-labelled Strep*Tactin were combined with 0.5 µg of HLA-A*02:01/STpp65$_{495\text{-}503}$ for 45 minutes at 4ºC in the dark.

1.1 Multimer staining optimization

A total of 1 x 10^6 human PBMCs were blocked with AB serum to avoid non-specific binding between MHC-multimers and FcR expressed on CD14 and CD19 cells (174). After washing, cells were incubated with 5 µL PM or 0.2 µg ST complex and incubated for 10 minutes at room temperature (RT) or 45 minutes at 4ºC in the dark, respectively. Both, multimer concentrations' used and incubation times were optimised previously in our laboratory (unpublished data). For multiparametric analysis, anti-CD3*HorizonV450 (Beckton Dickinson (BD), Heidelberg, Germany), anti-CD8* fluorescein isothiocyanate (FITC) and anti-CD45*peridin chlorophyll protein (PerCP-Cy5.5) monoclonal anti-human antibodies (Ab) (Biolegend, San Diego, CA) were added at RT for 15 minutes or 20 minutes at 4ºC in the dark, respectively. After lysis with Pharmlyse™ Lysing buffer (BD) to eliminate residual red blood cells, stained cells were acquired in a FACSCanto II flow cytometry (BD) equipment with FACSDiva 6.0 software (BD). A minimum of 500,000 events were acquired in all cases. Viable lymphocytes were gated based on their forward-scatter (FSC) and side-scatter (SSC) distribution and CD45 expression. $CD3^+CD8^+$ CTLs were gated and $PM^+CD3^+CD8^+$ and $ST^+CD3^+CD8^+$ percentages were determined.

The repeatability, correlation, sensitivity and the specificity of both multimers' techniques were evaluated in order to select the best strategy for the incorporation into clinical monitoring practice.

1.1.1 Repeatability analysis

Repeatability was examined by taking several measurements on a series of subjects. Then calculating the standard deviation between the repeated measures obtained for each subject. The variability observed between the independent values refers to the precision of the method. Therefore, in order to determine both the repeatability and the precision of PM and ST techniques, the frequency of CMV-specific $CD8^+$ T cells was evaluated in

6 HLA-A*02:01, CMV-seropositive and multimer-positive healthy donors per multimeric complex (n = 12). For each subject, six tubes were stained with either PM or ST multimers as described previously and percentages of $CD3^+CD8^+PM^+$ and $CD3^+CD8^+ST^+$ were determined.

1.1.2 Sensitivity assay

Correlation between both multimer staining was evaluated in 18 HLA-A*02:01 and CMV seropositive healthy volunteers. In order to compare the sensitivity of the quantification of CMV-specific T cells by each multimer (PM and ST), PBMCs from 5 HLA-A*02:01 CMV seropositive healthy donors were serially diluted with PBMCs from HLA-A2 negative healthy donors. CMV-specific cells were analysed and quantified as previously described according to $CD3^+CD8^+PM^+$ or $CD3^+CD8^+ST^+$ frequencies. The obtained results were compared with theoretical values of virus-specific T cells.

1.1.3 Specificity analysis

Specificity of PM and ST was tested in order to demonstrate that both multimers bind to CMV-specific CTLs with HLA-A*02:01 restriction. A total of 20 subjects with different HLA antigen expression and CMV-specific serological status combination were included: 5 HLA-A*02:01 and CMV seronegative, 5 HLA-A*02:01 negative and CMV seropositive, 5 HLA-A*02:01 negative and CMV seronegative and 5 HLA-A*02:01 positive and CMV seropositive. PBMCs were stained with PM and ST complex as described above and $CD3^+CD8^+PM^+$ and $CD3^+CD8^+ST^+$ frequencies were quantified.

2 CMV-specific $CD8^+$ T cells monitoring by multimer's staining

Both multimers were used in order to monitor CMV immune recovery. A total of 1 x 10^6 human AB serum-blocked PBMCs were incubated with either 5 µL PM or 0.2 µg ST complex and incubated for 10 minutes at RT or 45 minutes at 4ºC in the dark, respectively. For multiparametric analysis, anti-CD3*HorizonV450 (BD), anti-CD4*allophycocyanin (APC), anti-CD8*FITC and anti-CD45*PerCP-Cy5.5 monoclonal anti-human Ab (Biolegend) were added at RT for 15 minutes or 20 minutes at 4ºC in the dark, respectively. After lysing with Pharmlyse™ Lysing buffer (BD), stained cells were acquired in a FACSCanto II flow cytometry (BD) equipment with FACSDiva 6.0 software (BD). A minimum of 500,000 events were acquired in all cases. Cells with

lymphocyte morphology were gated based on their FSC and SSC distribution and $CD45^+$ expression. $CD3^+CD8^+$ CTLs were gated and $PM^+CD3^+CD8^+$ and $ST^+CD3^+CD8^+$ percentages were determined. Absolute numbers of multimer-staining cells were quantified using total lymphocyte counts from clinical laboratory analysis by using a cell counter (Beckman Coulter) and additional flow cytometry quantification of $CD8^+$ T-cell frequencies. The absolute number of multimer stained cells was calculated as the product of the following: (lymphocyte count)x(frequency of $CD3^+$ cells co-expressing $CD8^+$ in the lymphocyte $CD45^+$ gate)x(frequency of multimer stained cells in the $CD8^+$ T-cell subset).

3 CMV-specific $CD8^+$ T-cell detection by intracellular cytokine staining

To assess cytokine expression by CMV-specific $CD8^+$ T cells, 2 x 10^6 PBMCs from patients were incubated with 10 μg/mL of $pp65_{495-503}$ peptide (Proimmune, Oxford, UK) for 1 hour at 37ºC. Unstimulated PBMCs were used as negative control. Afterwards, cells were incubated in the presence of brefeldin A (10 μg/mL, Sigma-Aldrich, St Louis, MO, USA) for 5 hours at 37ºC. Following incubation, cells were washed with cold phosphate buffer saline (PBS) and then re-suspend in 200 μL of 0.02% EDTA (Sigma-Aldrich) for 15 minutes at 37ºC. Cells were then fixed using FACS Lysing solution/FACS Perm (BD) according to the manufacturer's instructions. Following fixation and permeabilization, cells were washed and stained with anti-CD3*HorizonV450 (BD), anti-CD8*FITC and anti-CD45*PerCP-Cy5.5 (Biolegend) and the intracellular marker anti-IFNγ*APC (BD) monoclonal anti-human antibodies for 30 minutes at RT. After washing in PBS, cells were analysed by flow cytometry. A minimum of 200,000 events were acquired in all cases. Cells with lymphocyte morphology were gated based on their FCS and SSC distribution and $CD45^+$ expression. The percentage of IFNγ-producing $CD8^+$ T cells was determined. The absolute number of IFNγ-staining cells was calculated as the product of the following: (lymphocyte count) x (frequency of $CD3^+$ cells co-expressing $CD8^+$ in the lymphocyte $CD45^+$ gate) x [(frequency of IFNγ-staining cells in the $CD8^+$ T-cell subset)$_{\text{activated cells}}$ - (frequency of IFNγ-staining cells in the $CD8^+$ T-cell subset)$_{\text{control cells}}$].

4 Detection of INFγ secreting cells by pentamer-positive cells

Cytokine secretion assay was developed in 9 patients. The assay was performed according to manufacturer's instructions (IFNγ Secretion Assay-Detection Kit, Miltenyi Biotec). Briefly, 1 x 10^6 PBMCs were stained with HLA-A*02:01/CMVpp65$_{495-503}$ PM*PE for 1 hour at 4ºC. After washing, cells were stimulated with 10 μg/mL of pp65$_{495-503}$ peptide for 2 hours at 37ºC. Unstimulated PBMCs served as negative control. Cells were then stained for IFNγ-secretion for 90 minutes at 37ºC. After washing, cells were stained with anti-CD3*Horizon V450, anti-CD8*FITC and anti-IFNγ*APC for 10 minutes at 4ºC. Cells were lysed before being acquired using flow cytometry. A minimum of 500,000 events were analysed in all cases. Viable lymphocytes were gated based on their FCS and SSC distribution. The percentage of IFNγ-production by PM^+ $CD8^+$ T cells was determined. The absolute numbers of $PM^+IFN\gamma^+$-staining cells were calculated as the product of the following: (lymphocyte count) x (frequency of $CD3^+$ cells co-expressing $CD8^+$ in the lymphocyte gate) x (frequency of $PM^+IFN\gamma^+$-staining cells in the $CD8^+$ T-cell subset).

B Phenotypic characterisation of CMV-specific $CD4^+$ T cells

1 Healthy donor characteristics

The study was approved by the West Midlands Research Ethics Committee. A total of 55 blood samples were obtained from healthy donors after informed consent was obtained. The donor cohort was split into three groups according to their age: young (18-40 years), middle aged (41-60 years) and older adults (over 60 years).

2 Blood sampling

PBMCs were isolated by density gradient centrifugation using Lympholyte^R-H cell separation media (Cedarlane laboratories, Canada) from 20-120 mL of heparin anticoagulated blood samples. Vials of 10x10^6 cells were cryopreserved in RPMI (Sigma, Aldrich) containing 20% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) (Fisher Scientific).

3 Anti-CMV antibodies detection in whole blood

CMV serostatus of healthy donors was evaluated by an 'in house' CMV-ELISA. Each plate was prepared with both negative control (mock lysate) and the CMV lysate (UV-inactivated) diluted 1/4000 carbonate-bicarbonate buffer (Sigma-Aldrich). Fifty microliters of diluted mock lysate or CMV lysate were added into each well, using a 96 well Nunc MAXISORP plate, and incubated overnight (o/n) at 4ºC in a sealed plated. The following day, the standard curve was made using serum from three pooled CMV seropositive healthy donors. The highest concentration was a 1/5 dilution from this positive mixture using PBS + 1% BSA (Sigma-Aldrich) + 0.05% TWEEN (Life Technologies) and subsequently, serial dilutions 1/4 were performed to provide a standard curve. Both samples and the standard curve were diluted 1/60 in PBS + 1% BSA + 0.05% TWEEN before addition to the plate. The ELISA plate was washed 3 times with washing buffer (PBS + 0.05% TWEEN) before adding the samples and the standard curve in a final dilution of 1/600 and incubated for 1 hour at RT. The plate was again washed 3 times before addition of 100µL of the anti-IgG-HRP conjugated detection antibody (Southern Biotech). A final dilution of 1/8000 of this antibody was incubated for 1 hour at RT. A further 3 wash steps were performed before addition of 100µL of the tetramethylbenzidine (TMB) substrate solution (eBiosciences). Substrate was incubated on the plate for 15 minutes at RT in the dark. The reaction was stopped by addition of 100µL of 1 M Hydrochloric acid (HCl) (Sigma-Aldrich). The plate was read at 450nm in a spectrophotometer within 20 minutes to prevent degradation of values obtained. IgG values over 10 were considered as seropositive for CMV.

4 Genomic DNA extraction and genotype HLA class-II

4.1 DNA extraction

Genomic DNA of CMV-seropositive donors was isolated from PBMCs according to manufacturer's instructions (GenElute™ Blood Genomic DNA Kit, Sigma-Aldrich). Briefly, PBMCs were incubated with proteinase K and lysis solution for 10 minutes at 55ºC to ensure efficient cell lysis and DNA release. After adding ethanol to the lysate, samples were transferred to the columns and centrifuged. The flow-through liquid was discarded and two consecutives washes were performed. To eliminate all the ethanol, the

column was air dried. Samples were eluted from the column by addition of RNAase-DNAase free water. DNA eluted from the column was quantified and purity assessed using the nanodrop spectrophotometer.

4.2 HLA class-II determination by PCR

Class-II PCR was performed as previously described (175). It consists of a method developed for DNA typing which uses sequence-specific primer (SSP) reactions to detect DRB1, DRB3 and DQB1 specificities. Our HLA of interest are DRB1*07:01, DRB3*02:02 and DQB1*06:02 (referred here as DR7, DR52b and DQ6, respectively). In Table 5 it is shown the primers used for each HLA-PCR. Donors' HLA restrictions are shown in Table 6.

Table 5. HLA class-II PCR conditions

HLA-type	Sense 5'→3'	Anti-sense 5'→3'
DR7	CCTGTGGCAGGGTAAGTATA	CCCGTAGTTGTGTCTGCACA
DR52b	GGAGTACCGGGCGGTAGAG	CGTATGCAGACACAACTACC
DQ6	TTTCGTGCTCCAGTTTAAGGC/ GACGTGGGGGTGTACCGC	CCGCGGAACGCCACCTC
DQ6	GGAGCGCGTGCGTCTTGTA	TGCACACCGTGTCCAACTC/ TGCACACCCTGTCCACCG

Table 6. Characteristics of the 55 healthy donors analysed.

Donor	No. of donors	HLA-type	Median age (range)
Young (20-40 years)	11	DR7	30 (25-39)
	3	DR52B	
	2	DQ6	
Middle aged (40-60 years)	9	DR7	49 (45-59)
	12	DR52B	
	5	DQ6	
Older adults (over 60 years)	9	DR7	73 (61-87)
	2	DR52B	
	2	DQ6	

5 CMV-specific CD4$^+$ T-cell cloning

Requirements for CD4$^+$ T-cell cloning:

- B-lymphoblastic cell lines with the proper HLA restriction (DR7, DR52b or DQ6)
- PBMCs from 3 different donors to serve as feeder cells
- CMV-specific CD4$^+$ T cells from CMV seropositive donors with DR7, DR52b and DQ6 restriction

5.1 B-Lymphoblastic cell line (LCL) generation

A total of 5 x 10^6 PBMCs from healthy donors were plated in 24-well plates containing RPMI media supplemented with 10% heat inactivated FCS, 1% L-glutamine, 1%penicillin/streptomycin and 1μg/mL of the immunosuppressor Cs-A (Sandoz Pharmaceuticals Inc, Washington, DC). Concentrated supernatant from B95-8 cultures, a human type I EBV-transformed B-cell line, was added for infection. Once B95-8 infected LCL were established, they were expanded into 25cm^2 flasks with LCL media (RPMI + 10% FCS).

5.2 PBMCs isolation

PBMCs were obtained by ficoll-hypaque density gradient centrifugation as described in section B2. PBMCs from 3 different healthy donors were filtered before isolating PBMCs. PBMCs from each buffy coat cell were re-suspended in 50mL of LCL media in a 75cm^2 flask. PBMCs were stimulated with 10μg/mL of phytohaemaglutinin (PHA) at 37ºC o/n. The following day, PBMCs were washed 3 times in LCL media and re-suspend in 30mL of media prior to γ-irradiation with 4000rads (CIS Bio International, IBL 437C). After irradiation, the pooled PBMCs were washed twice with LCL media and counted.

5.3 IFNγ capture of CMV-specific CD4$^+$ T cells

Before performing the selection of the IFNγ-secreted cells, a CD8$^+$ T-cell depletion was performed using anti-CD8 Dynabeads (Invitrogen). PBMCs were re-suspended in 2 mL of RPMI media in a 15mL tube and incubated with anti-CD8 dynabeads. It is assumed based on manufacturer's instructions that 1/3 of the cell population will be CD8$^+$ T cells, therefore 4 beads per cell are added to the tube prior to incubation at 4ºC. Then, 3 mL of

RPMI were added and the tube was placed in a magnet. The supernatant from this isolation was collected and a twice more with 5 mL of media. Resultant isolated cells were counted and pelleted at 2000g. After $CD8^+$ T-cell depletion, cells were stimulated in a 15mL tube with 5 mg/mL of $gB_{217-227}$, $pp65_{41-55}$ or $pp65_{489-503}$ peptides for 3 hours at 37ºC. After washing with cold RPMI media for 10 minutes at 4ºC and 2000g, cells were re-suspended in 80µL of cold media and stained for IFNγ production according to manufacturer's instructions (IFNγ Secretion Assay-Detection Kit, Miltenyi Biotec). Briefly, cells were incubated with 20µL of the IFNγ catch reagent for 5 minutes on ice. Then, cells were incubated lying horizontally on a rotator at 37ºC for 45 minutes with 10mL of warm media. After washing, cells were stained with the IFNγ detection antibody PE-conjugated for 10 minutes on ice. Cells were then washed and stained with anti-PE microbeads for 20 minutes at 4ºC. Cells were washed again, then re-suspended in 500µL of cold buffer (PBS supplemented with 0.5%BSA and 2mM EDTA). Subsequently, a MS column (Miltenyi Biotec) was placed in a magnetic field of a MACs separator. The column was rinsed with 500µL of cold buffer and the effluent was discarded. The magnetically labelled cells were applied to the column and allowed to pass through by gravity flow. Three washes with 500µL of cold buffer were performed and the collected effluent was the negative fraction. Afterwards, the column was removed from the magnet and 1mL of cold buffer was added to the column. By using a plunger, the retained cells were firmly flushed out from the column and the positive $CD4^+$ T cells specific for $gB_{217-227}$, $pp65_{41-55}$ or $pp65_{489-503}$ peptides were obtained.

5.4 Limiting dilution cloning

IFNγ-producing $CD4^+$ T cells following stimulation with CMV epitopes were sorted into 96-well plates by seeding at 0.3 cell/well or 3 cells/well. For each condition, ten 96-well V-bottom plates were prepared. A total of 10 x 10^6 peptide-pulse γ-irradiated LCL, 100 x 10^6 γ-irradiated pre-activated buffy cells and either 300 CMV-specific $CD4^+$ T cells ($gB_{217-227}$, $pp65_{41-55}$ or $pp65_{489-503}$ specific) for condition of 0.3 cells/well or 3000 CMV-specific $CD4^+$ T cells for condition of 3 cells/well were needed and make up to 100 mL with media (RPMI supplemented with 10% FCS, 5% human serum and 1% penicillin/streptomycin). Then, 100 µL per well were plated out and cells were incubated for 3 days at 37ºC. After 3 days of incubation, 100 µL of media (RPMI supplemented

with 10% FCS, 5% human serum, 1% penicillin/streptomycin, 30% supernatant from the MLA144 cell line (176, 177) and 50 U/mL recombinant IL-2) was added per well and clones were left growing during 3 weeks before testing. MLA144 cell line was a lymphoblastoid line established from a spontaneous lymphosarcoma of the gibbon. It supports the growth of T cells as it constitutively produces IL-2, IL-3 and TGFβ.

5.5 Specificity of CMV-specific $CD4^+$ T-cell clones

After 3 weeks in culture, specificity of potential CMV-specific $CD4^+$ T cell clones was assessed. For that purpose, autologous HLA-matched LCLs from CMV-seropositive donors were pulsed with either 5 μg/mL of the specific CMV-peptide ($gB_{217-227}$, $pp65_{41-55}$ or $pp65_{489-503}$) or DMSO solvent (the carrier agent for the CMV-peptides and therefore they would be the negative control) in 1mL of LCL media, for 2-3 hours at 37ºC. A total of 50 μL of cell suspension of each clone were transferred into V-bottom microtest plate wells. After washing, cells were split out into 4 wells and duplicates of each clone-analyses were performed (two wells with DMSO-pulsed LCLs and two wells peptide-pulsed LCLs). A total of 5 x 10^4 stimulated-LCLs/well were added. Cells were left over night (o/n) for IFNγ production.

5.5.1 INFγ production by CMV-specific $CD4^+$ T cells detection by ELISA.

IFNγ production of clones was evaluated by an in house IFNγ-ELISA. Briefly, ELISA plates were coated o/n with the primary antibody (IFNγ clone 2G1, Fisher Scientific) in a 1/1333 dilution with coating buffer (0.1M $NaHPO_4$ buffer, pH=9). Before addition of sample, plates were blocked with 50 μL of PBS + 1% BSA + 0.05% TWEEN per well for 1 hour. A standard curve was constructed by serial dilution from a known stock concentration of recombinant IFNγ. After washing 3 times, 50 μL of the IFNγ standard curve or the supernatant from the LCL-stimulated clones were added and incubated for 2 hours. After 6 times washes, the secondary antibody (IFNγ clone 2G1 biotin-labelled, Fisher Scientific) was added at a dilution of 1/1333 and incubated for one hour at RT. Six more washes were performed before incubation with streptavidin-peroxidase (Sigma-Aldrich) at a concentration of 1/1000 for 30 minutes. Plates were washed a final 3 times before incubation with 100μL per well with TMB substrate for 30 minutes. The reaction was stopped by addition of 100μL 1M HCl. Absorption was measured at 450nm by a spectrophotometer.

6 Detection of CMV-specific CD4$^+$ T cells by multimer staining

Tetramer

Class-II tetramer staining is more technically challenging than class-I as antigen-specific CD4$^+$ T cells are less common than antigen-specific CD8$^+$ T cells and the affinity between the T-cell receptor and HLA-peptide complex is generally lower. MHC class II tetrameric complexes were purchased from Benaroya Research Institute at Virginia Mason (Seatle, Washington). The three available tetramers were directed against the epitope DYSNTHSTRYV (217-227) of the CMV glycoprotein B (gB) in the context of HLA-DRB1*07:01 (DR7) restricted (27), or against the epitope AGILARNNLVPMVATV (489-503) of the CMV phosphoprotein 65 (pp65) in the context of HLA-DRB3*02:02 (DR52b) (26) restricted or against the epitope LLQTGIHVRVSQPSL (41-55) (25) of the CMV pp65 in the context of HLA-DQB1*06:02 (DQ6) restricted (Figure 8).

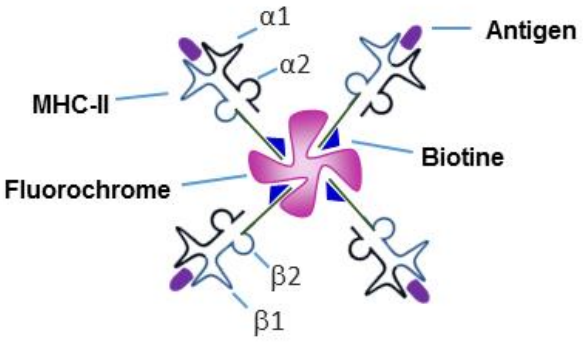

Figure 8. Structure of MHC class-II tetramers.

6.1 Tetramer sensitivity assay

To assess the sensitivity of the tetramers, 1 x 10^6 PBMCs from CMV-seronegative healthy donors DR7, DR52b or DQ6-restricted were mixed with decreasing amounts of CD4$^+$ T-cell clones (5%, 1%, 0.5%, 0.25%, 0.1%) specific for DYS, AGI or LLQ epitopes, respectively. In addition, the CD4$^+$ T-cell clone alone and PBMCs from the CMV-seronegative donor were also analysed. Cells were stained with LIFE/Dead fixable violet stain (Invitrogen) for 15 minutes at RT. After washing with PBS, cells were re-suspended

in 50 µL of human serum and incubated with 0.5 µL of the PE-conjugated MHC class II tetramer (DR7, DR52b or DQ6) for 1 h at 37ºC and 5% CO_2. Cells were then washed and subsequently co-stained with surface monoclonal anti-CD4*PerCP-Cy5.5 (eBioscience) antibody. All stained cells were acquired using a LSRII flow cytometer (BD) and all data were processed using Kaluza 1.3 software (Beckman Coulter). At least, 300,000 viable single lymphocytes were analysed in all cases. Viable single lymphocytes were gated based on their FSC and SSC distribution, from those, $CD4^+$ T cells were gated and $CD4^+tetramer^+$ percentages were determined.

6.2 CMV-specific $CD4^+$ T-cells phenotype

Donor PBMCs were thawed in warm RPMI, counted and re-suspended in PBS. A total of 1 x 10^6 PBMCs were stained with LIVE/Dead fixable violet stain (Invitrogen) for 15 minutes at RT. Cells were washed with PBS and re-suspended in 50 µL of human serum and incubated with 0.5 µL of the PE-conjugated MHC class II tetramer (DR7, DR52b or DQ6) for 1 h at 37ºC and 5% CO_2. Cells were then washed and subsequently co-stained with surface monoclonal anti-human antibodies (Table 7) for 15 minutes at 4ºC and then washed.

Table 7. Panels of CMV-specific $CD4^+$ T-cell phenotyping.

	mAb	**Company**		**mAb**	**Company**
phenotype	CD14*Pacific Blue	*(Biolegend)*	**exhausted**	CD14*Pacific Blue	*(Biolegend)*
	CD19*Pacific Blue	*(eBioscience)*		CD19*Pacific Blue	*(eBioscience)*
	CD3*AmCyan	*(BD)*		CD3*AmCyan	*(BD)*
	CD4*PerCP-Cy5.5	*(eBioscience)*		CD4*PE-CF594	*(BD)*
	CD27*APC-eFluor780	*(eBioscience)*		CD45RA*Alexa	*(Biolegend)*
	CD28*PE-Cy7	*(Biolegend)*		CD69*PE-Cy7	*(Biolegend)*
	CD45RA*Alexa Fluor700	*(Biolegend)*		CCR7*FITC	*(R&D)*
	CD45RO*PE-CF594	*(Biolegend)*		PD-1*PerCP-Cy5.5	*(Biolegend)*
	CD57*APC	*(Biolegend)*		Tim-3*APC	*(eBioscience)*
	CCR7*FITC	*(R&D)*			
cytotoxic	CD14*Pacific Blue	*(Biolegend)*	**regulatory**	CD14*Pacific Blue	*(Biolegend)*
	CD19*Pacific Blue	*(eBioscience)*		CD19*Pacific Blue	*(eBioscience)*
	CD3*AmCyan	*(BD)*		CD3*AmCyan	*(BD)*
	CD4*PE-CF594	*(BD)*		CD4*PE-CF594	*(BD)*
	CX3CR1*PerCP-Cy5.5	*(Biolegend)*		CD25*APC-Cy7	*(Biolegend)*
	FasL*AF488	*(AbD Serotec)*		CD127*PerCP-Cy5.5	*(BD)*
	GranzymeB*AF647	*(Biolegend)*		FoxP3*AF647	*(Biolegend)*
	Perforin*PE-Cy7	*(eBioscience)*			

For the detection of intracellular perforin and granzyme B, cells were fixed by addition of 4% paraformaldehyde/PBS for 15 minutes at RT in the dark after surface staining was performed. Anti-perforin*PE-Cy7 (eBioscience) and anti-granzyme B*AF647 (Biolegend) were added to the cells in the presence of 0.5% saponin (Sigma) in PBS, for 30 minutes at RT in the dark and then washed.

For the detection of intracellular FoxP3, cells were stained with surface markers and after washing they were fixed for 30 minutes using 1X Fix/Perm buffer (eBioscience). Cells were washed and permeabilized in Perm buffer (eBioscience) for 15 minutes in the dark and subsequently anti-FoxP3*AF647 (Biolegend) was added for 30 minutes at RT in the dark following one wash.

All stained cells were acquired using a LSRII flow cytometer (BD) and all data were processed using Kaluza 1.3 software (Beckman Coulter). A minimum of 300,000 viable single lymphocytes were analysed in all cases. Viable single lymphocytes were gated based on their FSC and SSC distribution, from those $CD3^+CD4^+$ T cells were gated and $CD3^+CD4^+$tetramer$^+$ percentages were determined.

7 Statistics analysis

Descriptive data are presented as mean with standard deviation for parametric data and median with median range for non-parametric data. Normality was studied with Kolmogorov-Smirnov test. Statistical analyses were performed by using parametric test (*t*-test) and non-parametric test (*U*-Mann Whitney and Kruskal-Wallis). To assess both the repeatability and sensitivity of each multimer technique two mixed effects models were performed by using statistical package R 2.13.1. Log-rank or Breslow tests were used for cumulative incidence of CMV reactivation during the first year after allo-HSCT. For correlation analyses the Spearman rank test was used. For correlation between the predicted positive values and the obtained with class II tetramers the Intraclass Correlation Coefficient (ICC) was used. For evaluation of both number of cells before and after reactivation and IFNγ production measured by ICS and the commercial kit, paired-Wilcoxon test was used. All *p*-values were two-tailed and values less than 0.05 were considered statistically significant. Statistical analyses were performed by using SPSS v.13 for Windows and GraphPad Prism 5.03.

IV) RESULTS

A Detection of Cytomegalovirus-specific CD8$^+$ T cells

1 Technique's optimization

As described in Materials and Methods (section A1), a total of 34 healthy volunteers were used in order to optimize the techniques (PM and ST) that are used for the monitoring of CMV-specific CD8$^+$ T cells immune recovery in allo-HSCT recipients. For that purpose, the repeatability, sensitivity and specificity of these two multimers used were evaluated.

1.1 Precision and repeatability analysis

The repeatability of each multimer technology was evaluated in order to determine the precision of both multimers staining. For that purpose, a total of 12 HLA-A*02:01/CMV-seropositive healthy donors were evaluated and six tubes per donor were stained with either PM or ST multimer. Frequencies of CMV-multimer$^+$CD8$^+$ T cells detected with both methodologies are shown in Table 8.

Table 8. Repeatability test that shows the percentage of CMV-specific CD8$^+$ T cells in HLA-A*02:01/CMV-seropositive healthy volunteers.

		Number of dilution						
	Donor	**1**	**2**	**3**	**4**	**5**	**6**	**SD.**
% of PM CMV pp65$_{495\text{-}503}$-specific CD8$^+$ T cells	**20***	0.994	1.044	1.002	0.912	1.015	0.973	0.045
	27	2.402	2.444	2.359	2.646	2.467	2.492	0.099
	28	0.872	0.851	0.812	0.884	0.829	0.846	0.027
	29*	0.946	0.863	0.818	0.956	0.909	0.865	0.054
	30	0.045	0.047	0.049	0.043	0.044	0.046	0.002
	31	0.524	0.694	0.655	0.652	0.650	0.672	0.060
% of ST CMV pp65$_{495\text{-}503}$-specific CD8$^+$ T cells	**16**	3.417	3.323	3.380	3.386	3.310	3.440	0.051
	20*	0.590	0.603	0.585	0.573	0.574	0.550	0.018
	26	0.755	0.703	0.725	0.701	0.722	0.700	0.021
	29*	0.432	0.461	0.487	0.424	0.461	0.482	0.026
	32	1.619	1.464	1.421	1.443	1.370	1.335	0.099
	34	1.930	1.830	1.797	1.835	1.855	1.741	0.063

*Those analyses were not performed at the same time, explaining the differences observed in the frequencies of CMV-multimer$^+$CD8$^+$ T cells.

As shown by the standard deviation (SD) of each measurement, both techniques are repetitive with a mean value of the SD of 0.048 (SD=0.033) for PM and 0.046 (SD=0.031) for ST. According to the mixed effect model results, both technologies showed low intra-

individual variability (σ_ε^2 = 3.2x10^{-3} and 3.0x10^{-3}, for PM and ST staining respectively), demonstrating that both methods could be considered equally precise.

1.2 Sensitivity study

Firstly, we wanted to compare both multimer staining and for this reason the correlation between both techniques was estimated. In this case, a total of 18 HLA-A*02:01/CMV-seropositive samples were analysed with both PM and ST multimers. The median frequencies of CMV-specific CD8$^+$ T cells were 1.990% (range: 1.161-3.587) and 1.933% (1.073-3.549) for PM and ST positive staining, respectively. Both multimers showed a strong correlation when more than 1% of antigen-specific cells was detected ($r_{Spearman}$ = 0.9422, p<0.001, Figure 9).

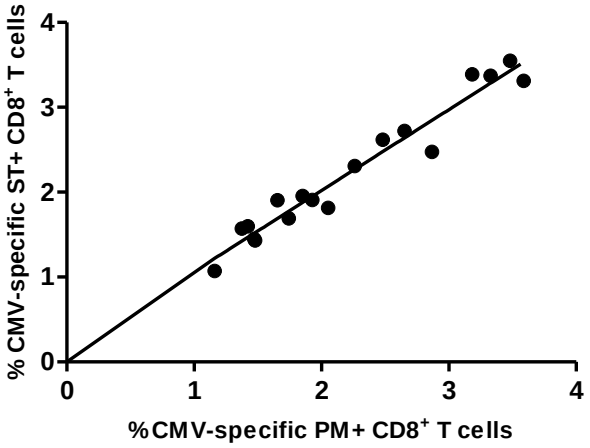

Figure 9. Correlation between pentamer and streptamer multimers of 18 HLA-A*02:01/CMV-seropositive donors.

As, during the first months post-HSCT, the proportion of virus-specific cells is quite low (71, 78), the detection of low frequencies of virus-specific cells is of special interest. For this reason, we evaluated the sensitivity of detection of each multimer. To define the sensitivity technique, PBMCs from HLA-A*02:01/CMV seropositive healthy donors were serially diluted with PBMCs from HLA-A2 negative healthy donors. A decrease of CMV-specific T cells by 50% in each subsequent sample was expected as 1:2 dilutions of the previous sample were prepared.

The detection levels of the smallest dilution (dilution nº7) were different between both techniques with a median frequency of PM positive cells of 0.049% (0.029-0.158) compared to 0.015% (0.003-0.037) of ST positive cells (Table 9). It is noticeable that PM staining provided higher values than ST staining mainly starting at dilution nº4, where frequencies of multimer positive $CD8^+$ T cells were equal or lower than 0.117 % (range: 0.028-0.130) (according to the theoretical data). However, the positive results given by the PM technique differed from the theoretical ones. By using a mixed effects model which takes into account the technique, the dilution and the individual, and looking at the differences between the observed values and the expected ones, it was shown that the ST technique gave closer values to the expected theoretical ones than PM, and those differences were significant ($p < 0.001$). Mean values of the differences between the observed values and the theoretical ones obtained by each technique at each dilution point are represented in Figure 10.

Table 9. Multimer positive $CD8^+$ T cells detected in each dilution, where 1 is the undiluted fraction and 7 is the highest dilution.

		Dilution number						
Donor	**Multimer**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
16	PM	0.948	0.599	0.289	0.163	0.097	0.081	0.039
	Theoretical data	0.841	0.371	0.175	0.085	0.042	0.021	0.010
	ST	0.889	0.492	0.196	0.116	0.064	0.03	0.015
21	PM	0.885	0.583	0.505	0.242	0.210	0.184	0.156
	Theoretical data	1.008	0.504	0.252	0.126	0.063	0.032	0.016
	ST	0.721	0.399	0.208	0.103	0.061	0.030	0.014
24	PM	0.742	0.340	0.359	0.168	0.140	-	0.058
	Theoretical data	0.742	0.162	0.063	0.028	0.014	0.007	0.003
	ST	0.699	0.257	0.177	0.088	0.051	0.024	0.014
25	PM	1.162	0.820	0.591	0.555	0.287	0.302	0.158
	Theoretical data	1.003	0.503	0.252	0.126	0.063	0.032	0.016
	ST	1.213	0.903	0.461	0.231	0.118	0.069	0.037
26	PM	0.708	0.244	0.156	0.087	0.073	0.026	0.029
	Theoretical data	1.016	0.514	0.259	0.130	0.065	0.032	0.016
	ST	0.913	0.407	0.198	0.115	0.077	0.032	0.013
27	PM	0.759	0.387	0.241	0.108	0.095	0.066	0.039
	Theoretical data	1.000	0.497	0.248	0.124	0.062	0.031	0.015
	ST	0.939	0.679	0.591	0.154	0.085	0.037	0.030

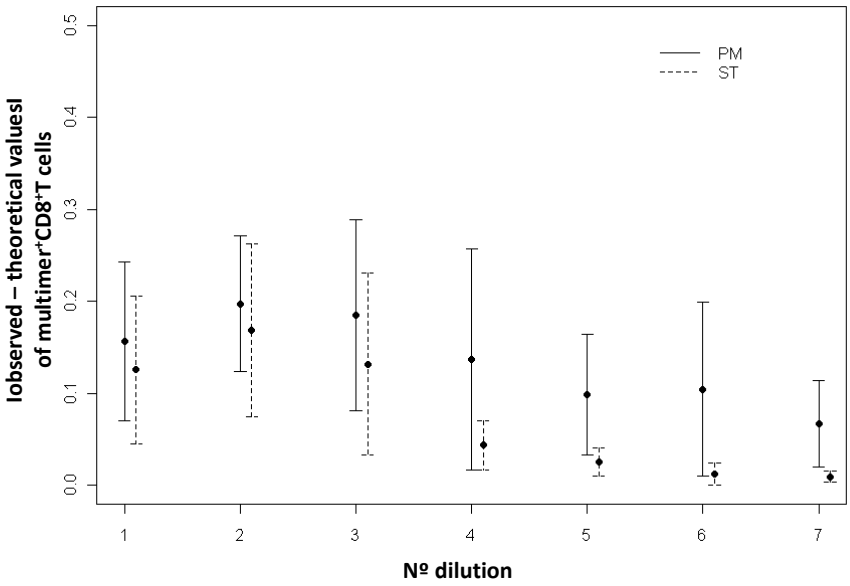

Figure 10. Sensitivity assay of CMV-specific multimer$^+$ CD8$^+$ T cells. Peripheral blood mononuclear cells (PBMCs) from 6 HLA-A*02:01/CMV seropositive healthy donors were serially diluted with PBMCs from 6 HLA-A2 negative healthy donors. Mean values of the absolute differences between the observed values and the theoretical ones of multimer$^+$CD8$^+$T cells obtained by each technique (PM and ST) in each dilution point where 1 is the undiluted fraction and 7 the highest dilution are shown.

This fact could be explained by the observed differences in the median fluorescence intensity (MFI) between PM and ST staining. ST multimer gave considerably brighter staining than achieved with PM technology with higher MFI values (Figure 11). The median MFI values of the undiluted fraction of CMV-specific CD8$^+$ T cells were 19255 (10067 - 41913) and 8191 (5700 - 17127) for ST and PM respectively (Table 10). Similar differences were observed with all dilutions (data not shown), allowing a good identification of CMV-specific T cells with the ST technique but not with PM multimer in those individuals with small percentage of these cells. The MFI of staining with ST was more than 2.4-fold brighter than with PM (p=0.009) (Table 3).

Therefore, PM technology despite detecting higher amounts of cells is less accurate than ST.

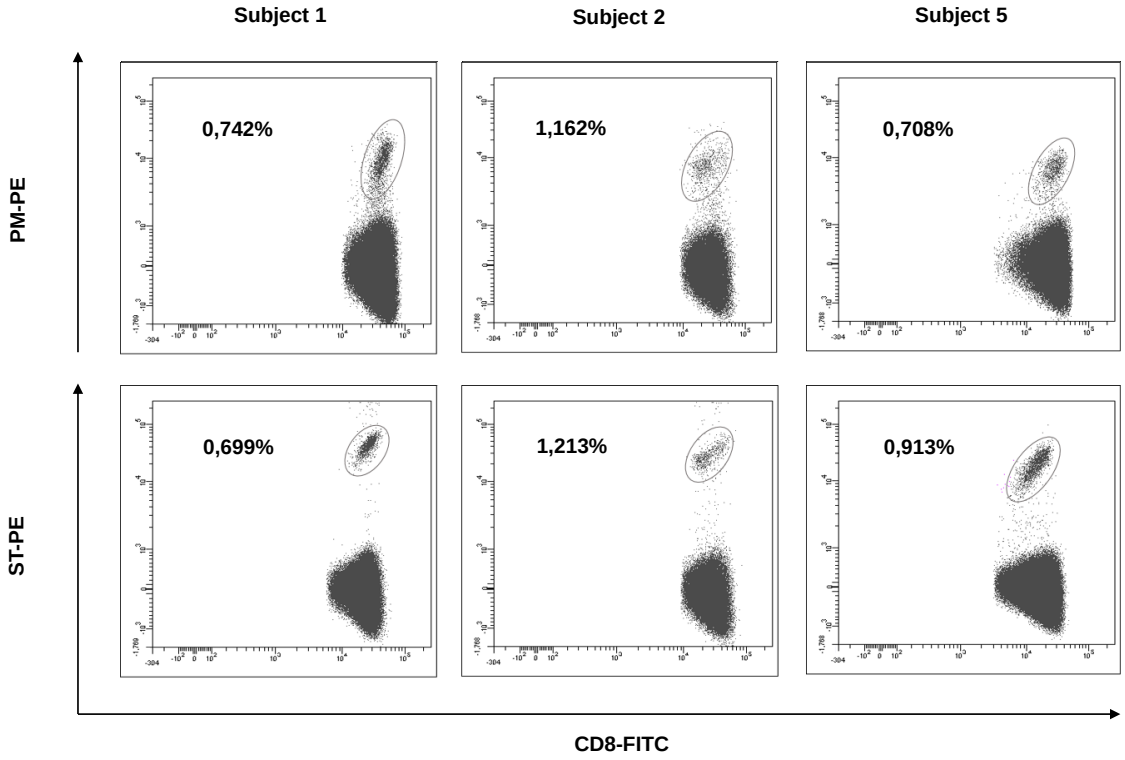

Figure 11. Representative bi-exponential dot plots of the undiluted CMV-specific $CD8^+$ T cells obtained with both multimers, pentamer (PM) (A) and streptamer (ST) (B) from subjects 1, 2 and 5. Frequencies of multimer-staining cells were gated within the $CD8^+$ T-cell subset co-expressing $CD3^+$ in the lymphocyte gate $CD45^+$. At least 100,000 events were analysed within the $CD8^+$ T-cell population. Median Fluorescence Intensity (MFI) values were obtained from 6 subjects using those dot plots.

Table 10. Median Fluorescence Intensity values of the undiluted CMV-specific $CD8^+$ T cells stained with each multimer.

Donor	Multimer	MFI	Range
16	PM	6401	2062-15344
	ST	18855	4799-51671
21	PM	5700	1086-35791
	ST	10067	1389-38944
24	PM	8979	3171-25099
	ST	41913	13231-104183
25	PM	7438	3356-26937
	ST	25024	9412-71634
26	PM	17127	5520-39665
	ST	19655	2537-60857
27	PM	8944	3897-32523
	ST	13167	2350-38944

1.3 Validation of multimer specificity

In order to confirm previous data, specificity of each multimer was analysed in 20 healthy volunteers with different CMV serostatus and HLA characteristics (Table 11). In all 5

HLA-A*02:01/CMV-seropositive healthy donors, similar values of CMV-specific $CD8^+$ T cells were detected with both multimers with no statistic differences (median of 3.705% (0.407-6.880) and 3.474% (0.347-7.154), for PM and ST positive cells respectively). However, when comparing the staining in non-HLA-A*02:01/CMV-seronegative (n=5), non-HLA-A*02:01/CMV-seropositive (n=5) and HLA-A*02:01/CMV-seronegative (n=5) volunteers, higher background levels could be detected with the PM technique. The median frequency of positive cells observed with the PM technology was 0.035% (0.000-0.136), whereas a median of 0.007% (0.000-0.021) positive cells was detected with the ST technology (p = 0.003).

These results confirm that ST multimer technology is more specific for the capture of HLA-A*02:01-restricted $CMV_{495-503}$-specific $CD8^+$ T cells compared to PM multimer staining.

Table 11. Percentage of $CD8^+$ T cells that bind to pentamer or streptamer multimers in HLA-A*02:01 and non- HLA-A*02:01 CMV-seropositive or negative healthy donors.

Volunteer	HLA-A	CMV serostatus	Percentage of pentamer positive cells (%)	Percentage of streptamer positive cells (%)
1	A*32, A*36	positive	0.010	0.013
2	A*24, A*33	positive	0.018	0.003
3	A*26, A*31	positive	0.006	0.009
4	A*01, A*03	positive	0.008	0.000
5	A*29, A*68	positive	0.050	0.000
6	A*03, A*24	negative	0.005	0.009
7	A*01, A*24	negative	0.089	0.021
8	A*29, A*29	negative	0.076	0.007
9	A*03, A*30	negative	0.023	0.005
10	A*11, A*23	negative	0.007	0.006
11	A*02:01, -	negative	0.136	0.007
12	A*02:01, A*24	negative	0.047	0.008
13	A*02:01, A*24	negative	0.071	0.015
14	A*02:01, A*03	negative	0.074	0.003
15	A*02:01, -	negative	0.000	0.000
16	A*02:01, A*29	positive	1.886	1.700
17	A*02:01, -	positive	4.746	4.248
18	A*02:01, A*01	positive	0.407	0.347
19	A*02:01, A*24	positive	3.705	3.474
20	A*02:01, A*30	positive	6.880	7.154

2 Immune reconstitution of CMV-specific $CD8^+$ T cells after allogeneic haematopoietic stem cell transplantation

2.1 Patient demographic data

A total of 25 HLA-A*02:01 recipients who underwent allo-HSCT were studied for T-cell immune reconstitution during one year following transplant. One patient gave up the study two months after transplantation and only those data were taken into account. Median follow-up was 12 months (2-12). Shorter follow-up times were primarily because of early deaths (n=5).

A total of 13 (52%) males and 12 (48%) females were transplanted with a median age of 42 years (24-65). The diagnosis of the patients were as follows: 4 recipients with lymphoma (Hodgkin and no Hodgkin, 16%), 18 patients had leukaemia (72%), two people presented myelodysplastic syndrome (8%) and only one person had an inherit disease, Fanconi anaemia (4%).

Eleven of 25 patients received a sibling transplant, two patients received a transplant from a haploidentical relative and a total of 12 patients received transplant from an unrelated donor. A total of 11 recipients (44%) received MA conditioning treatment whereas 14 patients (56%) received RIC therapy. Those patients whose donors were unrelated received *in vivo* T-cell depletion with either Alentuzumab or Campath® or ATG. Most patients (96%) received PBSCs as stem cell source whereas only one patient received BM as stem cell source.

A total of 16 CMV-seropositive recipients received a graft from a CMV-seropositive donor whereas 8 CMV-seropositive recipients received a transplant from CMV-seronegative donors. Only one patient of 25 was CMV-seronegative before transplantation and received a graft from a CMV-seropositive donor.

Acute GvHD was developed in 11 patients (44%), and chronic GvHD (grade II-IV) was developed in eleven of 25 patients that were treated with metilprednisone (1mg/Kg/12h). Demographic and clinical characteristics of these patients are shown in Table 12.

Neutrophil engraftment (more than 0.5 x 10^9 neutrophils/L) occurred at a median of 20 days (12-30) and platelet engraftment (more than 20 x 10^9 platelets/L) at 16 days (10-37).

Table 12. Characteristics of the 25 patients analysed

Characteristics	Value
Age (median (range))	42 (24-65)
Sex (M/F)	13/12
Diagnosis	
NHL	2
HL	2
CLL	1
AML	13
ALL	4
MDS	2
Fanconi	1
Donor/recipient CMV serostatus	
Positive/Positive	16
Negative/Positive	8
Positive/Negative	1
Donor type	
Sibling	11
Haploidentical relative	2
Unrelated	12
Stem cell source	
Bone marrow	1
Peripheral Blood	24
Conditioning treatment	
Myeloablative conditioning regimen	11
Bu/Cy± Campath or ATG	2
Cy/TBI±Campath	9
Non-myeloablative conditioning regimen	14
Flu/Mel±Campath	5
Cy/F±Campath	3
Flu/Bu±Campath or ATG	6
GvHD prophylaxis	
CsA + MTX	21
CsA + MMF	2
CsA + metilPDN	1
MMF +MTX	1
Acute GvHD grade	
0 to I	2
II to IV	9
Steroid therapy	
Yes	7
No	17

M = male; F = female; NHL = non-Hodgkin lymphoma; HL = Hodgkin; CLL = chronic lymphocytic leukemia; AML = acute myeloid leukemia; ALL = acute lymphoblastic leukemia; MDS = myelodysplastic syndrome; Bu = busulfan; Cy = cyclophosphamide; ATG = anti-thymocyte globulin; TBI = total body irradiation; Flu = fludarabine; Mel = melfalan; CsA = cyclosporine; MTX = methotrexate; MMF = mycophenolate mofetil; PDN = prednisone

As complications following transplant occur during the first three months after transplantation it is important to asses T-cell immune reconstitution during that period of time. Normal median values of total lymphocyte counts (0.9 - 4.0x10^3 lymphocytes/μL)

were reached by day 60 in most recipients (median 0.950 x10^3 lymphocytes/μL (0.1-5.3)) as well as $CD8^+$ T cells (>50 $CD8^+$ T cells/μL) and $CD4^+$ T cells (>100 $CD4^+$ T cells/μL) (Table 13).

Table 13. Median values of total lymphocyte count, $CD8^+$ and $CD4^+$ T cells during the first three months post-transplant.

day	Median lymphocytes (cells/μL)	range	Median $CD8^+$ (cells/μL)	range	Median $CD4^+$ (cells/μL)	range
30	0.300	0.000-3.800	23.546	0.000-2858.816	63.711	0.000-524.322
60	0.950	0.100-5.300	65.922	0.000-3927.618	133.972	0.000-642.600
100	0.800	0.100-3.300	171.120	0.053-2176.499	148.335	0.000-755.918

2.2 CMV reactivation

According to CMV reactivation status we have identified two groups of patients. Sixteen of 25 recipients (64%) experienced CMV reactivation at least once in the year follow up whereas 9/25 patients (36%) did not experience any CMV reactivation during 12 months following transplant. The median time to first CMV reactivation was 39 days (1-96). No differences were found in the engraftment day between both groups with a median of 21 days (17-29) and 18 days (12-25) for patients without and with CMV reactivation, respectively.

The cumulative incidence of CMV reactivation during the first year following allo-HSCT was not influenced by either HLA compatibility (p=0.145) or conditioning treatment (MA *vs*. RIC; p=0.119). On the contrary, the incidence of CMV reactivation was significantly influenced by HSCT donor type (unrelated *vs* sibling; p=0.036) and T-cell depletion in conditioning treatment (p=0.044). Both data are related as all patients that received a transplant from an unrelated donor, they also had *in vivo* T-cell depletion in the conditioning treatment.

The patient that was CMV-seronegative before transplantation and received a graft from a CMV-seropositive donor became infected with CMV after HSCT and recurrence of CMV reactivations were developed. This patient was not taking into consideration when donor CMV serostatus was analysed in order to see its influence on CMV reactivation showing that CMV reactivation was not related to donor CMV serostatus (p=0.630).

Five of the 8 CMV-seropositive patients whose donor was CMV-seronegative reactivated CMV as evidenced by positive PCR at a median of 33 days (1-39) after HSCT (Table 14). These 5 patients received antiviral therapy with GCV, VGC or foscarnet. Only 1 patient required cidofovir therapy because of failure of normal antiviral treatment. None of these patients developed CMV disease. One patient who did not develop CMV viremia was treated with cidofovir because of BK virus infection. This patient died due to GvHD.

Ten of 16 CMV-seropositive recipients from a CMV-seropositive donor developed CMV antigenemia after HSCT; the PCR became positive at a median of 41 days (10-96) after HSCT (Table 14). All these patients received antiviral therapy with GCV, VGC or foscarnet. Two of these recipients were also treated with cidofovir because of BK virus infection. Only one patient developed CMV disease. Four patients died before the year follow up due to transplant-related complications.

Table 14. CMV antigenemia, CMV disease and time of follow up of the 25 allo-HSCT recipients

Patient	Onset of CMV antigenemia*	No. of antiviral treatment courses*	Onset CMV disease*	Follow up*
1	41	3	-	365
2	38	2	-	365
3	-	0	-	365
4	49	3	-	365
5	-	0	-	365
6	-	0	-	365
7	33	5	-	365
8	17	2	-	157
9	25	2	-	365
10	-	0	-	95
11	10	3	-	365
12	-	0	-	365
13	96	2	-	365
14	47	3	-	365
15	39	4	-	365
16	-	0	-	365
17	56	3	143	365
18	41	2	-	346
19	57	1	-	193
20	10	2	-	206
21	-	0	-	328
22	-	0	-	338
23	30	2	-	279
24	-	0	-	60
25	-3	5	-	365

*day after haematopoietic stem cell transplantation

Absolute counts of $CD4^+$ and $CD8^+$ T cell subsets of recipients with no CMV-DNAemia and patients with CMV reactivation were analysed at 30, 60, 100, 200 and 300 days post-transplant, as some patients reactivated CMV after day +100 post-transplantation. No statistics differences in the median number of either $CD8^+$ or $CD4^+$ T cells were observed between both groups (Figure 12).

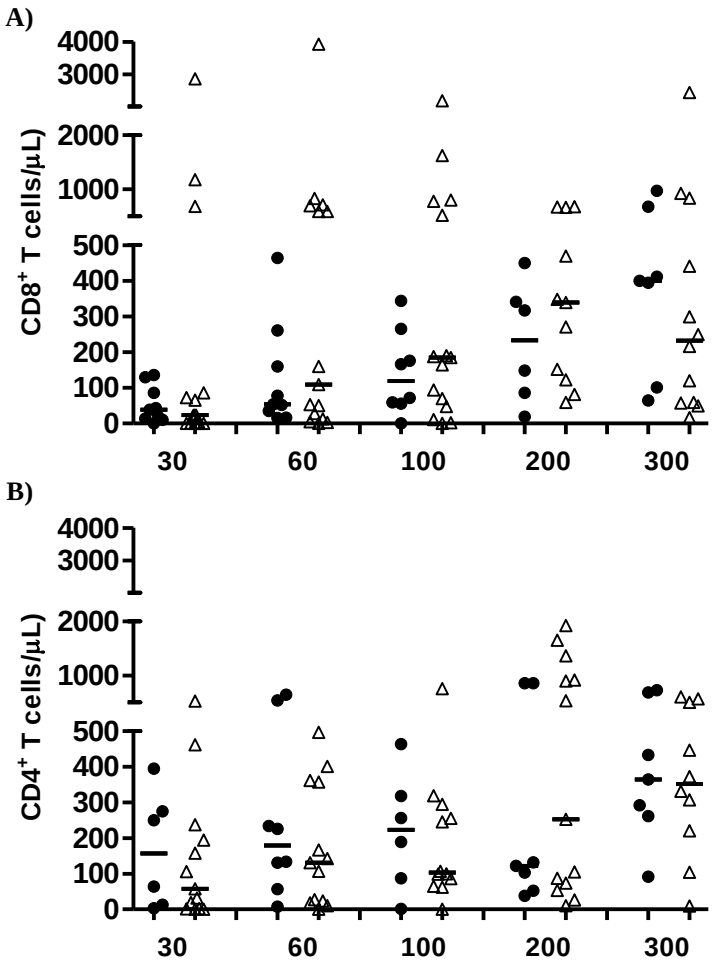

Figure 12. Levels of $CD8^+$ (A) and $CD4^+$ (B) T cells at days 30, 60, 100, 200 and 300 after allogeneic haematopoietic stem cell transplantation for recipients without (black circles) or with (white triangles) detectable CMV reactivation.

2.3 Monitoring of cytomegalovirus immune recovery by multimer technology

2.3.1 CMV-specific T-cell reconstitution and first CMV reactivation

CMV-specific immune reconstitution following transplant can be measured by the use of multimer technology. In our study, we have used streptamer technology, as it has been previously shown to be an accurate and precise tool, in order to monitor CMV-specific

$CD8^+$ T-cell recovery after allo-HSCT. We have observed, in our cohort of patients that CMV-multimer$^+$$CD8^+$ T-cell levels ranged from 0.000 to 678.384 cells/μL during the year follow up after allo-HSCT in patients with and without CMV-DNAemia. CMV-CTL absolute counts and total $CD3^+CD8^+$ absolute numbers showed a good correlation, regardless of reactivation status during the first 3 months following transplant (Table 15). Besides, a correlation between CMV-CTLs and total $CD3^+CD4^+$ absolute counts was also observed (Table 15).

Table 15. Correlation between CMV-CTL and T-cell subsets during the first 3 months after HSCT.

	Correlation	**CD4 & CD8**	**CD4 & CMV-CTL**	**CD8 & CMV-CTL**
Day 30	$r_{Spearman}$	0.833	0.614	0.564
	p	*<0.001*	*0.005*	*0.005*
Day 60	$r_{Spearman}$	0.621	0.443	0.621
	p	*0.003*	*0.044*	*0.001*
Day 100	$r_{Spearman}$	0.715	0.689	0.777
	p	*0.001*	*<0.001*	*0.002*

By the first month following transplant, most patients have started to recover the immune system and it is considered as early post-transplant period. If there is CMV infection, it normally occurs within the first three months post-transplant and therefore, CMV-specific immune recovery must be monitored. In our cohort of individuals, the onset of CMV-specific immune response was delayed on recipients with CMV reactivation compared to patients without reactivation (median days 48 and 26, respectively), and this delay tend to be significant (p=0.061). CMV reactivations occurred between days 30 and 60 after transplantation in most recipients, only 6 recipients reactivated before day +30. At that time point, no differences were found on CMV-specific T-cell levels between patients without reactivation and patients with CMV-DNAemia after 30 days of transplantation (median of 0.123 CMV-CTLs/μL (0.000-0.630) and 0.070 CMV-CTLs/μL (0.000-26.838), respectively; p=0.738) (Figure 13). Therefore, these results have shown that, in our cohort of recipients, the number of CMV-specific $CD8^+$ T cells one month after allo-HSCT seems to not protect against CMV reactivation.

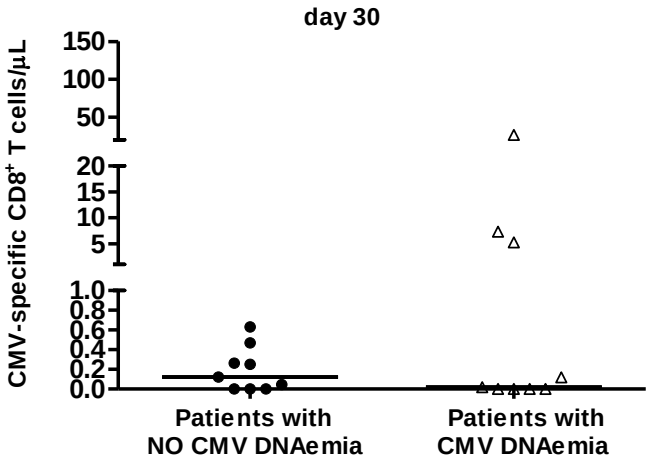

Figure 13. CMV-specific $CD8^+$ T cells of patients without (black circles) and with (white triangles) CMV reactivation at day 30 after allogeneic stem cell transplantation.

The majority of CMV reactivations occurred before day +60 following transplant (median 39 days (1-96)). The patient that reactivated at day +96 was probably because of immunosuppressive treatment due to GvHD. On the contrary to what was observed at day +30 following transplant, two months after allo-HSCT there was a significant increase on CMV-multimer$^+$ $CD8^+$ T cells in patients with CMV reactivation compared with recipients with no CMV DNAemia (median of 3.584 CMV-CTLs/µL (0.000-135.892) and 0.890 CMV-CTLs/µL (0.000-4.530), respectively; p=0.027) (Figure 14). These results show that CMV reactivation induces a CMV-specific $CD8^+$ T-cell expansion. Median number of CMV-specific $CD8^+$ T cells of both groups during the first three months are shown in Table 16.

Table 16. Median CMV-specific $CD8^+$ T-cell levels during the first three months after allo-HSCT in patients without and with CMV reactivation.

	Patients without CMV reactivation		**Patients with CMV reactivation**	
day	**Median CMV-specific $CD8^+$ T cells (cells/µL)**	**range**	**Median CMV-specific $CD8^+$ T cells (cells/µL)**	**range**
30	0.123	0.000-0.630	0.020	0.000-26.838
60	0.890	0.000-4.530	2.540	0.000-135.892
100	0.503	0.000-11.248	2.798	0.000-27.837

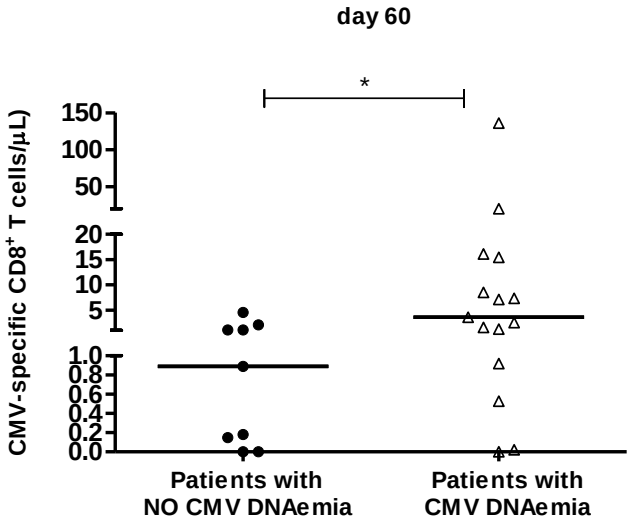

Figure 14. CMV-specific $CD8^+$ T cells of patients without (black circles) and with (white triangles) CMV reactivation at day 60 following allogeneic stem cell transplantation. *p<0.05.

2.3.2 CMV-specific $CD8^+$ T-cell responses and antiviral treatment

In our cohort of recipients, we have observed two different groups within patients that have CMV reactivation (n=16) when looking at the time of antiviral treatment. Twelve of 16 recipients were treated for less than 3 weeks (considered as short antiviral treatment) when CMV was detected by PCR whereas the rest (n=4) required prolonged antiviral treatment (more than 3 weeks). We have observed that patients with short antiviral treatment have a significant increase on CMV-CTLs after first CMV reactivation what does not occur in patients with prolonged antiviral treatment (Table 17 and Figure 15A). Patients that did not have an expansion on CMV-CTLs after CMV reactivation received a transplant from a CMV-seronegative donor (Figure 15B and C). These observations corroborate the increase on CMV-CTLs observed at day +60 in patients with CMV-DNAemia compared to recipients without CMV reactivation.

Table 17. Number of CMV-specific $CD8^+$ T cells detected before and after first CMV reactivation in patients with short (less than 3 weeks) and prolonged (more than 3 weeks) antiviral treatment.

Time of antiviral treatment	**CMV-CTL before first CMV reactivation (cells/µL)**	**range (cells/µL)**	**CMV-CTL after first CMV reactivation (cells/µL)**	**range (cells/µL)**	***p value***
< 3 weeks	0.006	0.000-36.710	4.685	0.000-135.892	*0.042*
> 3 weeks	0.000	0.000-0.000	0.011	0.000-0.432	*0.364*

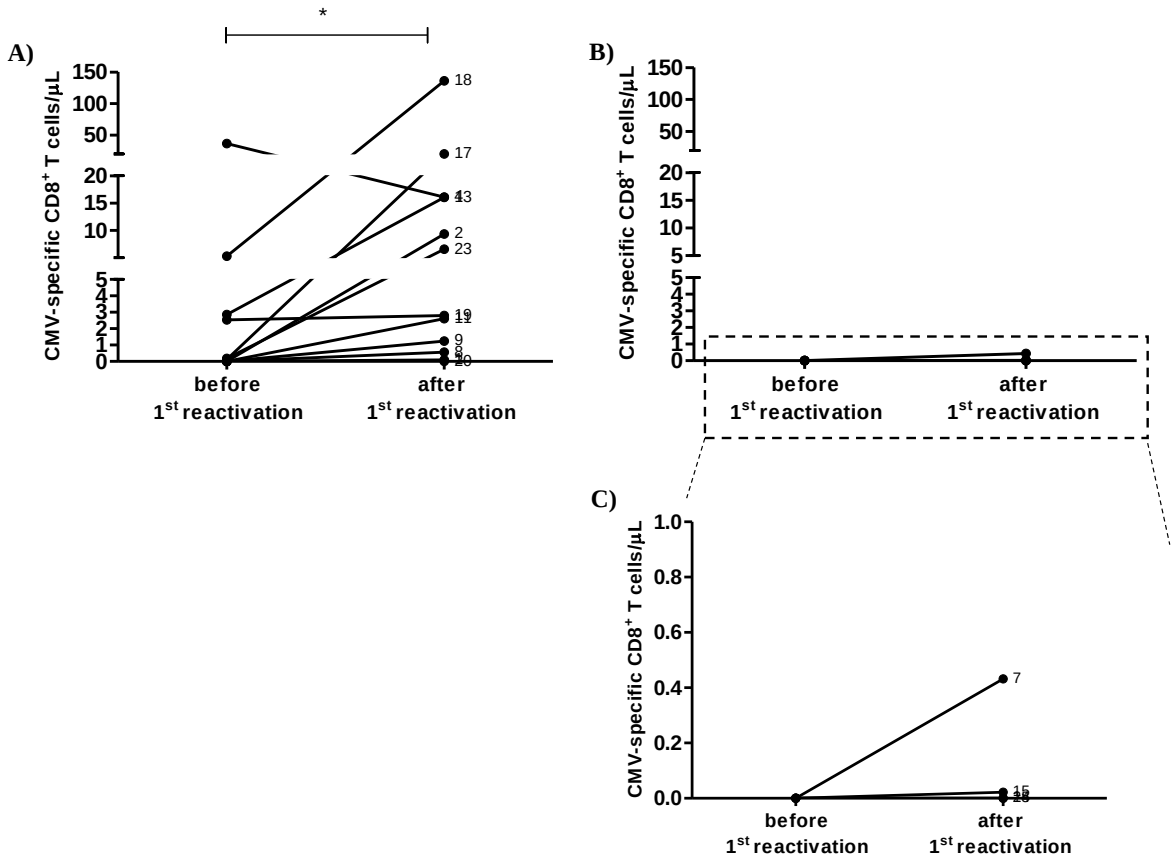

Figure 15. CMV-specific CD8$^+$ T cells detected before and after first CMV reactivation in patients that received antiviral treatment for less than 3 weeks (A) and recipients that required prolonged antiviral treatment to clearance CMV viremia (B and C). *p<0.05.

When comparing multimer positive CD8$^+$ T cells between both groups after the first CMV reactivation, there is a significant increase of CMV-specific CD8$^+$ T cells in patients with short antiviral treatment compared to recipients requiring long antiviral therapy time (median of 4.685 multimer$^+$CD8$^+$ T-cells/µL (0.000-135.892) and 0.011 multimer$^+$CD8$^+$ T-cells/µL (0.000-0.432), respectively; p=0.015) (Figure 16).

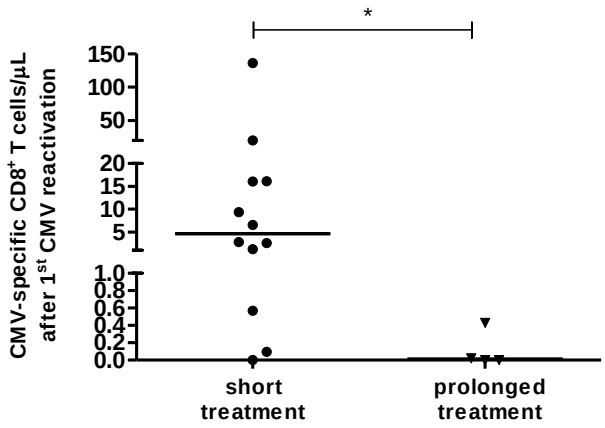

Figure 16. Multimer positive $CD8^+$ T cells detected after first CMV reactivation in patients with short antiviral treatment and therefore CMV-CTL expansion and patients with prolonged antiviral treatment and no expansion of CMV-specific T cells. *p<0.05.

It is interesting to mention that patients with no expansion of CMV-CTLs have significantly longer duration of CMV-DNAemia compared to patients with CMV-CTLs expansion after the first CMV reactivation (median of 27.5 days (8-34) *vs.* 7 days (3-18), respectively; p=0.012). Besides, they also had significant higher levels of CMV copies/mL when comparing to recipients that expanded CMV-CTLs (median of 29122 CMV-copies/mL (4790-103089) and 2697 CMV-copies/mL (915-14210), for patients without and with CMV-specific $CD8^+$ T-cell expansion respectively; p=0.011).

It has been previously shown that both $CD4^+$ and $CD8^+$ T-cell recovery was achieved in most patients by day +60 after allo-HSCT. However, there were differences within patients that experienced CMV reactivation. Recipients that received short antiviral treatment had higher levels of both $CD4^+$ and $CD8^+$ T cells compared to patients requiring prolonged antiviral therapy (130.809 $CD4^+$ T cells/µL (0.000-497.131) and 109.431 $CD8^+$ T cells/µL (0.000-3927.618) *vs.* 9.889 $CD4^+$ T cells/µL (0.000-142.919) and 4.961 $CD8^+$ T cells/µL (0.000-696.958), respectively). No statistical differences were found due to the low number of samples in the cohort of patients that received prolonged antiviral treatment. This group of recipients started to recover normal values of $CD8^+$ T cells by day +200 post-transplantation (median of 152.187 $CD8^+$ T cells/µL (81.93-339.240)) whereas normal $CD4^+$ T-cell levels started at day +300 following transplant (median of 307.619 $CD4^+$ T cells/µL (9.502-446.380)).

According to previous studies, recovery of T-cell function by day +100 after transplantation is important in order to avoid future CMV reactivations or disease and even mortality. For this reason, median numbers of CMV-CTLs by day +100 were studied in the cohort of patients that reactivated the virus. At this time point, patients with short antiviral treatment and expansion of specific-cells had significantly higher levels of CMV-CTLs compared to patients without CMV-specific T-cell expansion (4.376 cells/μL (0.064-27.837) and 0.000 cells/μL (0.000-0.000), respectively; $p < 0.001$) (Figure 17).

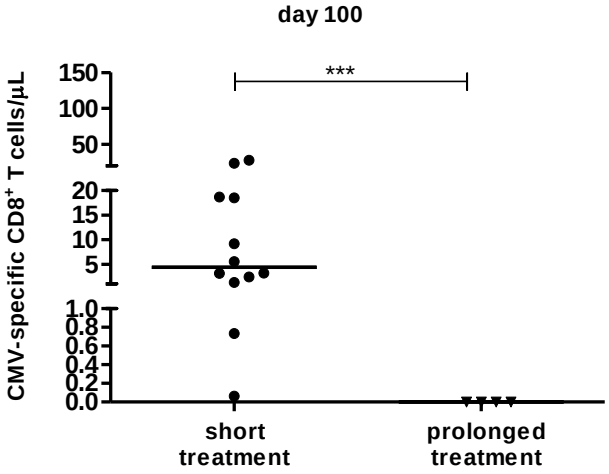

Figure 17. CMV-specific $CD8^+$ T-cell levels at day +100 following HSCT in recipients with CMV expansion after CMV reactivation and therefore short antiviral treatment and patients with no CMV expansion after CMV reactivation requiring prolonged antiviral therapy. ***p<0.001.

2.3.3 CMV-CTL expansion and multiple CMV reactivations

We were further interested in studying whether or not this CMV-CTL expansion was able to protect to multiples CMV reactivations in the recipient, defined by 2 or more distinct episodes of viremia separated by a negative PCR result.

Five of the 12 patients that have CMV-CTL expansion (and therefore short antiviral treatment) had only 1 CMV reactivation before day +100 after transplantation. The number of virus-specific T cells after the first and unique CMV reactivation was significantly higher in those patients compared with recipients that have several CMV reactivations (n=7) (median of 16.038 CMV-CTLs/μL (2.800-135.892) and 1.240 CMV-CTLs/μL (0.000-16.110), for patients with one or more than one CMV reactivation, respectively; p=0.042). Besides, examination of general T-cell reconstitution data showed

that patients with only 1 CMV reactivation had also rapid recovery of total $CD4^+$ cells (≥100 cells/μL) and total $CD8^+$ cells (≥50 cells/μL) (Table 18 and Figure 18).

Table 18. Median numbers of cells after the first CMV reactivation within patients with short antiviral treatment.

	1 reactivation before day +100	**Several reactivations before day +100**	***p value***
CMV-CTLs(cells/μL) (range)	16.038 (2.800-135.892)	1.240 (0.000-16.110)	*0.042*
$CD4^+$ T cells (cells/μL) (range)	375.121 (165.913-497.131)	27.548 (0.000-361.810)	*0.049*
$CD8^+$ T cells (cells/μL) (range)	630.038 (67.660-3927.618)	23.546 (0.000-588.665)	*0.028*

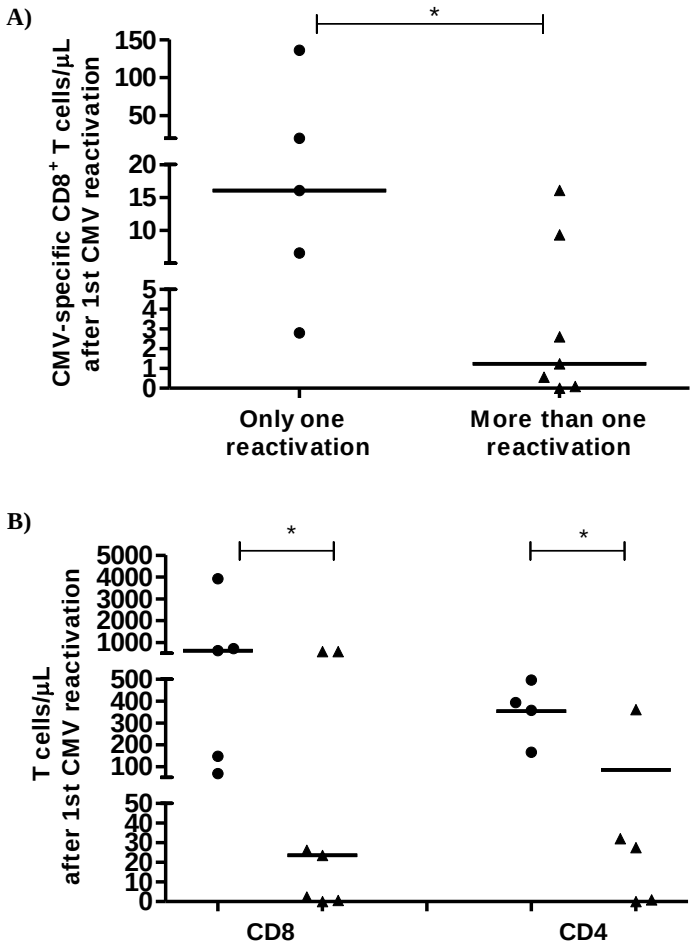

Figure 18. Median values of CMV-CTLs (A) and $CD4^+$ and $CD8^+$ T cells (B) after the first CMV reactivation in patients with only 1 reactivation (black circles) or multiple CMV reactivations (black triangles) within patients with short antiviral treatment. *p<0.05.

As previously mentioned, there were 7 of 12 patients within the CMV-CTL expansion group and therefore short antiviral treatment, who experienced more than 1 CMV reactivation. A total of 4 patients developed 2 CMV reactivations before day +100 after allo-HSCT and three recipients had 3 episodes of CMV-DNAemia during the first three months post-transplant. Levels of multimer$^+$CTLs after the second CMV reactivation were higher in patients that experienced only 2 episodes of CMV-DNAemia compared with recipients that had 3 CMV reactivations (median of CMV-CTL cells after 2nd CMV reactivation of 8.754 cells/μL (1.596-24.550) and 1.236 cells/μL (0.169-3.708), respectively). However, no statistical differences were observed, possibly due to the low number of samples analysed.

We were interested in knowing if there was a protective value that those recipients could achieve after CMV reactivation which protects against future CMV reactivations. For that purpose, we analysed the CMV-specific CD8$^+$ T-cell levels reached after the last CMV reactivation of those recipients. Patients that have only one CMV reactivation (n=5) have a median of 16.038 multimer$^+$CTLs/μL (2.800-135.892) after reactivation, recipients that experienced two episodes of CMV-DNAemia (n=4) achieved 8.754 multimer$^+$CTLs/μL (1.596-24.550) after the second CMV reactivation, and patients who had three reactivations (n=3) had 11.840 multimer$^+$CTLs/μL (1.348-23.490) after the third episode of CMV-DNAemia (Table 19). Our results have shown that the minimum cell number of virus-specific T cells reached after CMV reactivation was 1.348 multimer$^+$CTLs/μL and then no more CMV reactivations were detected.

Table 19. Median numbers of CMV-specific CD8$^+$ T cells after last reactivation of patients that have 1, 2 or 3 CMV reactivations.

Number of CMV reactivations	Median of multimer$^+$CTL (cells/μL)	Range (cells/μL)
1 (n=5)	16.038	2.800-135.892
2 (n=4)	8.754	1.596-24.550
3 (n=3)	11.840	1.348-23.490

As it was previously shown, four patients did not have an increase on CMV-CTLs numbers after CMV reactivation. Those patients received antiviral treatment for more than 3 weeks (**¡Error! No se encuentra el origen de la referencia.**B and C).

Patient 7 (D-/R+) experienced 5 CMV reactivations (day +33, +47, +90, +132 and +181), which were treated by VGC. No CMV-CTLs were detectable at these time points. This

patient developed GvHD at day 92 and was treated with high levels of immunosuppressive drugs which impaired CMV-specific $CD8^+$ T-cell recovery. By day +201 this patient had 0.028 CMV-CTLs/μL and did not reactivate CMV thereafter.

Patient 15 (D-/R+) had 4 CMV reactivations (day +39, +87, +130 and +186) that were treated with VGC. This patient had 0.25 CMV-CTLs/μL by day +130 but they did not seemed able to protect against another CMV reactivation on day +186.

Patient 25 (D-/R+) became infected before transplantation and was initially treated with GCV, then foscarnet the day of transplantation. Due to failure of pre-emptive treatment it was necessary to use cidofovir. Following this, the patient developed 4 more CMV reactivations (day +55, +131, +179 and +277) that were treated by either VGC or foscarnet. None CMV-CTLs could be detected until the final reactivation on day +277 (0.432 CMV-CTLs/μL). The number of CMV-specific $CD8^+$ T cells increased and no more CMV reactivations were observed.

Patient 14 (D+/R-) had 4 CMV reactivations that were treated with VGC. This patient received ATG and the lymphocyte count was very low until day +100 (less than 200 lymphocytes/μL). The patient then began to recover their lymphocyte function and after the last CMV reactivation on day +159, CMV-CTLs could be detected.

2.3.4 CMV-specific $CD8^+$ T-cell recovery in patients with CMV reactivation

The results obtained with multimer technology have led us to describe two different patterns in our group of patients according to CMV-specific $CD8^+$ T-cell recovery and antiviral treatment.

The most frequent pattern observed is the one where recipients experienced CMV-specific $CD8^+$ T-cell expansion after CMV reactivation and therefore requiring short period of antiviral treatment (n=12). There is a significant increase of those cells by day 60 compared to patients with no CMV-DNAemia as it has previously been described. The median number of CMV-specific $CD8^+$ T cells observed in each time point after transplantation was 5.570 cell/μL (0.568-8.340) (median numbers of virus-specific cells during the year follow up after allo-HSCT) (Figure 19A).

A second pattern where patients did not have an expansion of CMV-specific $CD8^+$ T-cell levels after episodes of CMV-DNAemia and therefore required prolonged time of antiviral therapy (n=4) (Figure 19C and D). These patients had very low levels of CMV-

specific CD8$^+$ T cells following transplant (median of 0.035 CMV-CTLs/μL (0.000-0.195) during one year following allo-HSCT) and experienced CMV reactivations after 3 months post-transplant.

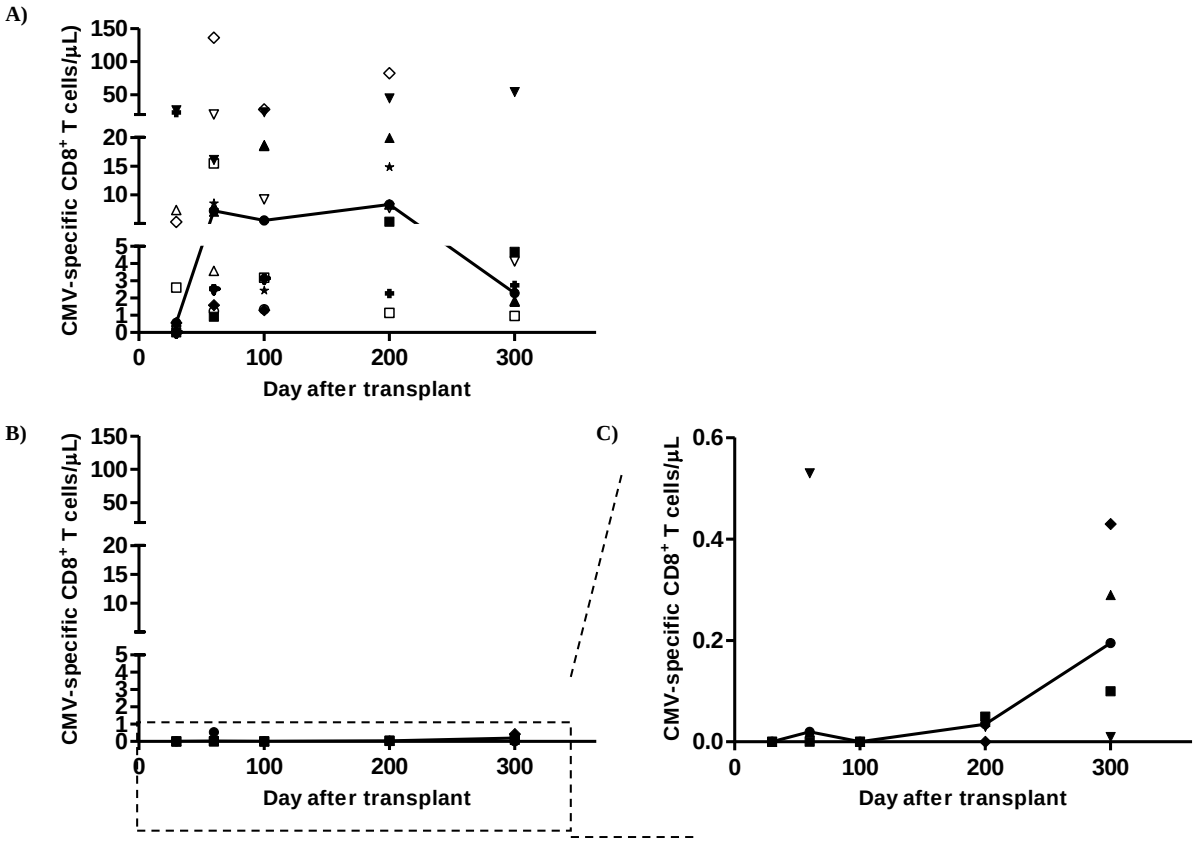

Figure 19. Different patterns observed in the cohort of individuals that reactivated CMV. Patients that had CMV-CTLs expansion after CMV reactivation and required short times of antiviral treatment (n=12) (A) and patients with no CMV-specific CD8$^+$ T cells after CMV reactivation requiring prolonger cumulative antiviral therapy (n=4) (B and C). The black line indicates the median of CMV-CTL levels at days +30, +60, +100, +200 and +300 post-transplan

2.4 Functional activity of virus-specific CD8$^+$ T cells

Another methodology widely used to monitor CMV-specific immune reconstitution is by measuring the production of cytokines upon stimulation with virus peptides, virus lysate or viral protein.

Functional assays were performed in order to assess functional activity of virus-specific CD8$^+$ T cells by monitoring intracellular IFNγ production upon stimulation with CMV-pp65$_{495\text{-}503}$ peptide. We have selected IFNγ because it is the main cytokine secreted by cytotoxic CD8$^+$ T cells upon activation by antigens (178). Due to the low number of cells for some patients, only 19 of 25 recipients could be monitored.

Six of the 19 recipients did not experience CMV-DNAemia during the year follow up whereas a total of 13 patients developed CMV reactivation after HSCT. As mentioned previously, most CMV reactivations occurred between day 30 and 60 after transplantation, only 5 patients reactivated CMV before day +30. At that time point, significant differences were found on *in vitro* IFNγ production levels between patients without CMV reactivation (n=6) and recipients with CMV-DNAemia a month after transplantation (n=8) (median of 0.094 IFNγ$^+$CD8$^+$ T-cells/μL (0.000-1.026) and 0.000 IFNγ$^+$CD8$^+$ T-cells/μL (0.000-0.000), respectively; p=0.022) (Figure 20).

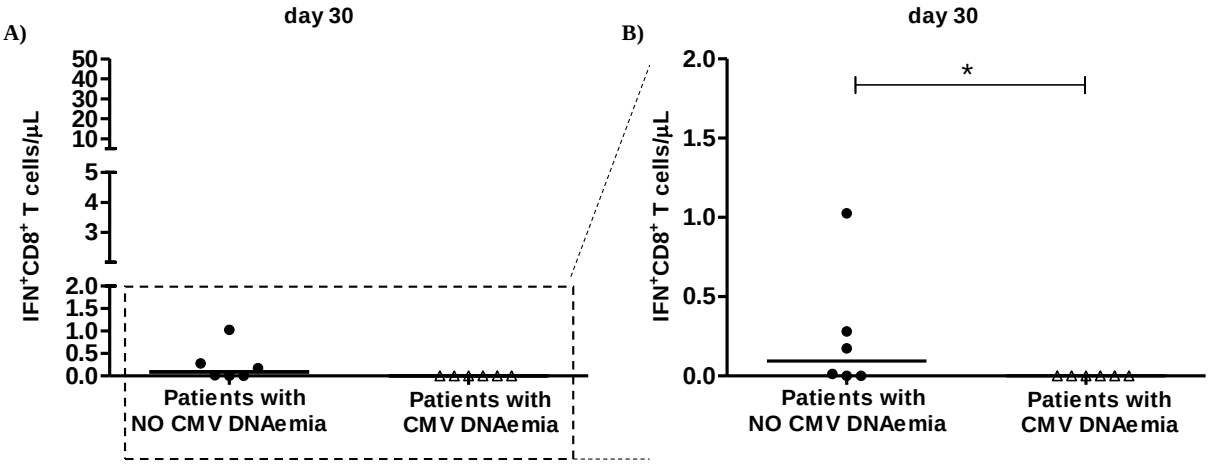

Figure 20. INFγ production levels by CD8$^+$ T cells upon *in vitro* stimulation with CMV-pp65$_{495\text{-}503}$ peptide in patients without (black circles) and with (white triangles) CMV reactivation at 30 days after transplantation.*p<0.05.

By day 60 post allo-HSCT the majority of CMV reactivations had occurred within patients that experienced CMV-DNAemia. After two months following transplant, there

was an increase of *in vitro* IFNγ-production by $CD8^+$ T cells when comparing patients without CMV reactivation and recipients with CMV-DNAemia (median of 0.305 $IFN\gamma^+CD8^+$ T-cells/μL (0.000-5.830) and 2.108 $IFN\gamma^+CD8^+$ T-cells/μL (0.000-40.02), respectively); however this difference was not significant, probably due to the high dispersion of the data (Figure 21).

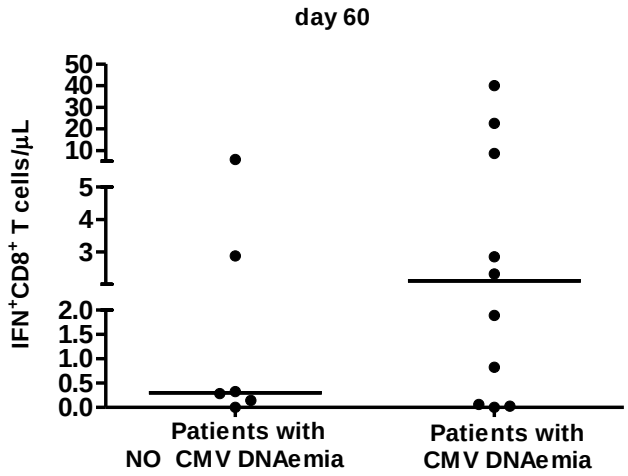

Figure 21. INFγ production levels by $CD8^+$ T cells upon *in vitro* stimulation with CMV-$pp65_{495\text{-}503}$ peptide in patients without (black circles) and with (white triangles) CMV reactivation at 30 days after transplantation.

2.4.1 Functional CMV-specific $CD8^+$ T cells and antiviral treatment

According to our previous classification in relation to antiviral treatment duration, a total of 10/13 recipients that experienced CMV reactivation were treated for less than 3 weeks, defined previously as short treatment, whereas 3 patients received prolonged antiviral therapy, defined by more than 3 weeks of treatment. Similar to multimer$^+$CTLs, we observed that patients with short antiviral therapy have a significant increase on *in vitro* IFNγ-production after the first CMV-reactivation (p=0.008) whereas recipients with prolonged antiviral therapy do not expanded IFNγ-producing $CD8^+$ T cells (Table 20 and Figure 22). There is no statistical analysis for patients with prolonged antiviral treatment as only data for two patients could be analysed.

Table 20. Number of IFNγ producing cells detected before and after first CMV reactivation in patients with short antiviral treatment (less than 3 weeks) and prolonged therapy (more than 3 weeks).

Time of antiviral treatment	Median of IFNγ production before CMV reactivation (cells/µL)	range (cells/µL)	Median of IFNγ production after CMV reactivation (cells/µL)	range (cells/µL)	*p value*
< 3 weeks	0.000	0.000-2.855	2.403	1.474-40.015	*0.008*
> 3 weeks	0.000	0.000-0.000	0.011	0.000-0.022	-

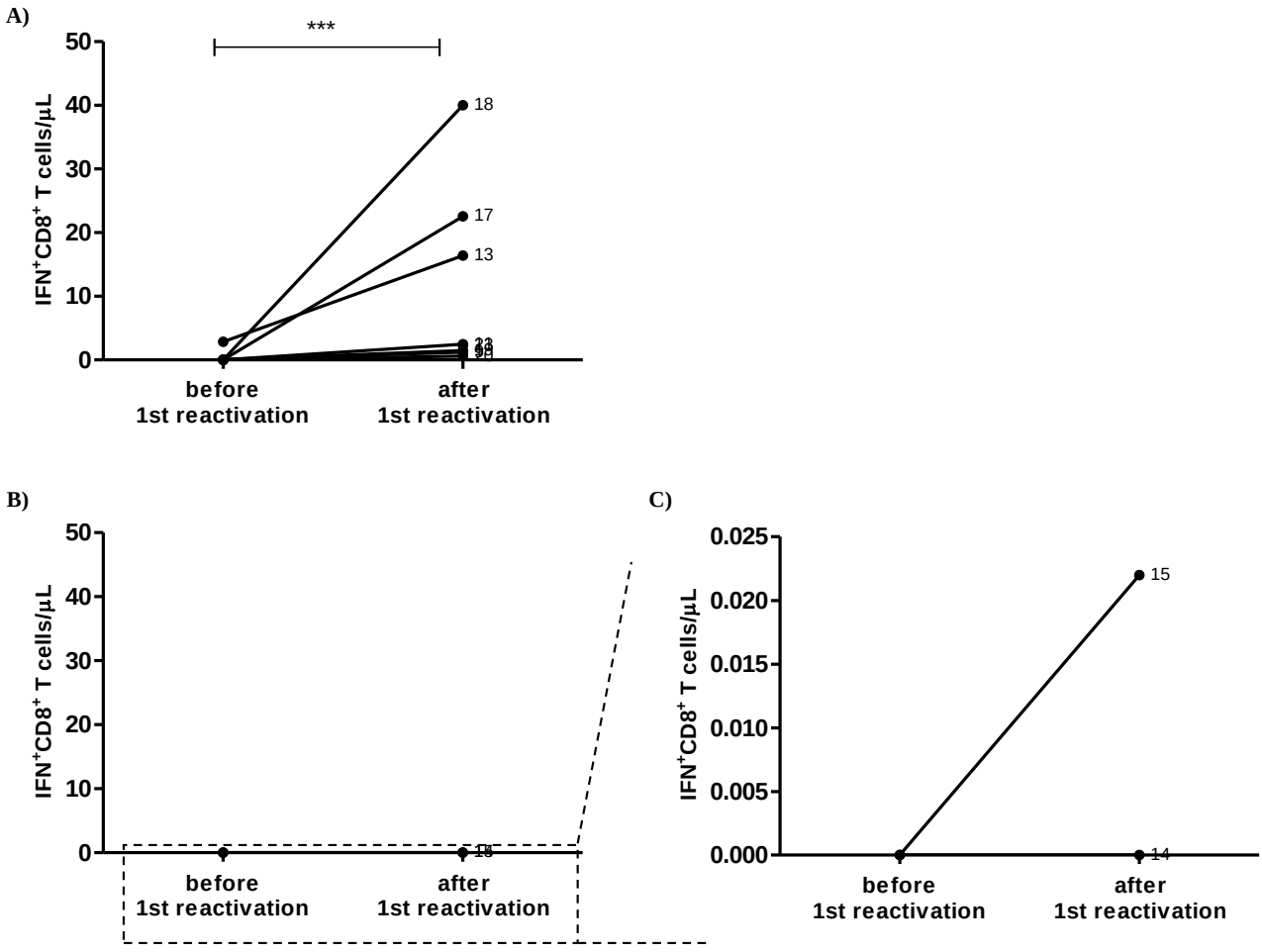

Figure 22. IFNγ production by $CD8^+$ T cells upon stimulation with the CMV-$pp65_{495-503}$ peptide before and after CMV reactivation in patients that received short antiviral therapy when CMV was detected (A) and recipients requiring prolonged antiviral treatment (B and C). ***p<0.001

When comparing the *in vitro* production of IFNγ levels between both groups after the first CMV reactivation, a significant increase in the number of cells responding to CMV-antigen in patients with short antiviral treatment was observed, when compared to

recipients requiring long antiviral therapy time (median of 2.403 IFNγ^{+}CD8^{+} T-cells/μL (1.474-40.015) and 0.011 IFNγ^{+}CD8^{+} T-cells/μL (0.000-0.022), respectively; p=0.037) (

Figure **23**).

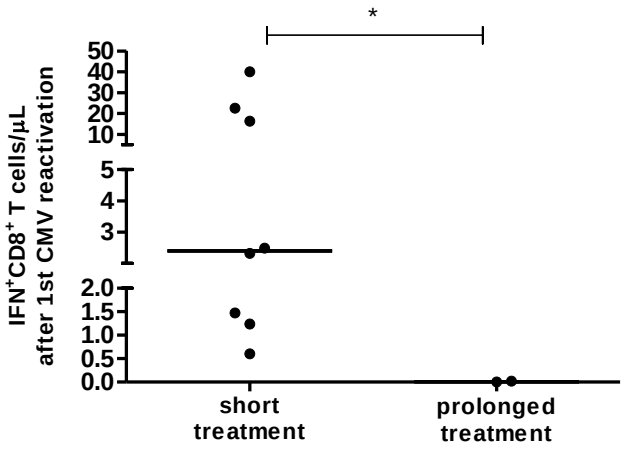

Figure 23. IFNγ levels detected after first CMV reactivation in patients with short and prolonged antiviral treatment. *p<0.05.

Functionality of CD8^{+} T cells upon *in vitro* stimulation with CMV-pp65$_{495\text{-}503}$ peptide was studied at day +100 after transplantation as CMV-specific immune recovery at that time point is important in order to avoid future CMV reactivations. When looking at patients that experienced CMV-DNAemia, it is observed that recipients with short antiviral treatment had significant higher levels of IFNγ compared to patients with prolonged antiviral treatment at day +100 after HSCT (median 2.743 IFNγ^{+}CD8^{+} T cells/μL (1.474-14.110) and 0.000 IFNγ^{+}CD8^{+} T cells/μL (0.000-0.000), respectively; p=0.011) (Figure 24).

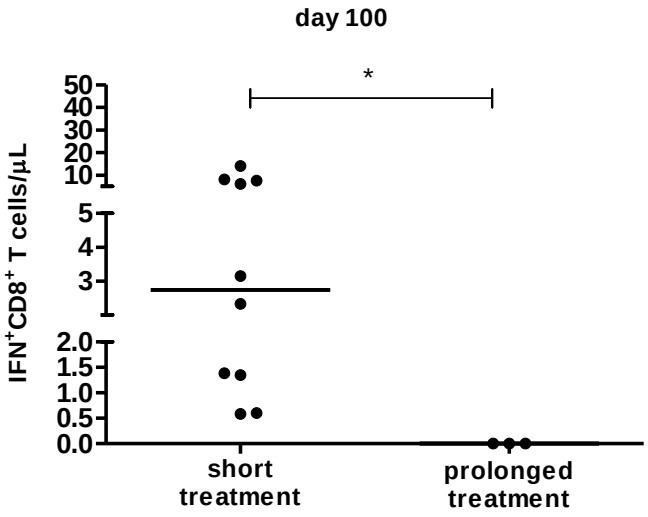

Figure 24. *In vitro* IFNγ production by $CD8^+$ T cells after stimulation with CMV-pp65 peptide at day +100 following transplant in recipients with short and prolonged antiviral treatment. *p<0.05.

2.4.2 IFNγ-production and multiple CMV reactivations

Five of the 10 patients that experienced an increase in *in vitro* IFNγ production levels after CMV reactivation and therefore, short antiviral treatment, had only one episode of CMV-DNAemia. We have compared the levels of IFNγ that those recipients produce upon *in vitro* stimulation after the first CMV reactivation with patients that have more than 1 reactivation. After the first and unique CMV reactivation, the levels of IFNγ detected were 16.380 $IFN\gamma^+CD8^+$ T cells/µL (1.474-40.015). On the contrary, patients with more than one CMV reactivation had 1.241 $IFN\gamma^+CD8^+$ T cells/µL (0.604-2.486). No statistical differences were observed due to the low number of samples as only 3 patients with multiple reactivations could be analysed (Figure 25).

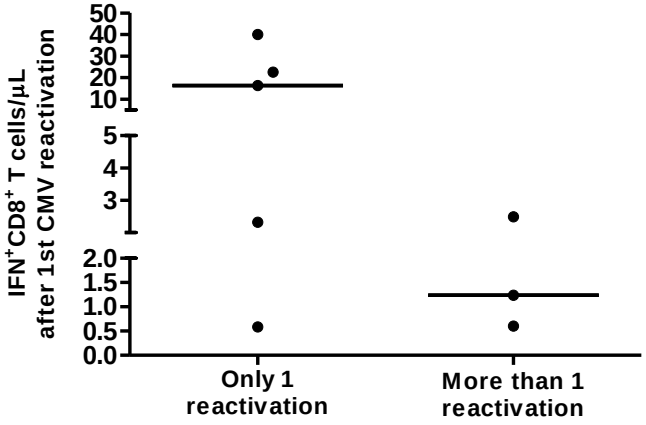

Figure 25. IFNγ$^+$CD8$^+$ T-cell levels detected after first CMV reactivation in patients with 1 or more than one CMV reactivation and receiving short antiviral treatment.

A total of 2 patients within those 10 patients that have short antiviral treatment experienced two CMV reactivations before day +100 after allo-HSCT and 3/10 had three episodes of CMV-DNAemia during that time. We have evaluated the levels of IFNγ production after the last CMV reactivation in those recipients to see if there is a protective value to further reactivations. Patients that have only one CMV reactivation (n=5) have a median of 16.380 IFNγ$^+$CD8$^+$ T-cells/µL (1.474-40.020) after reactivation, recipients that experienced two episodes of CMV-DNAemia (n=2) achieved 7.179 IFNγ$^+$CD8$^+$ T-cells/µL (5.744-8.613) after the second CMV reactivation, and patients who had three reactivations (n=3) had 1.385 IFNγ$^+$CD8$^+$ T-cells/µL (1.290-20.70) after the third episode of CMV-DNAemia (Table 21). These results have shown that the minimum cell number of IFNγ$^+$ production by CD8$^+$ T cells reached after CMV reactivation was 1.290 cells/µL and then no more CMV reactivations were detected.

Table 21. Median numbers of CMV-specific CD8$^+$ T cells after last CMV reactivation of patients that have 1, 2 or 3 CMV reactivations.

Number of CMV reactivations	Median of IFNγ$^+$CD8$^+$ T cells (cells/µL)	Range (cells/µL)
1 (n=5)	16.380	1.474-40.020
2 (n=2)	7.179	5.744-8.613
3 (n=3)	1.385	1.290-20.700

As mentioned previously, three of 19 patients analysed for functional activity did not have increased numbers of IFNγ producing $CD8^+$ T cells after CMV infection and received antiviral treatment for more than 3 weeks.

Patient 7 (D-/R+) experienced 5 CMV reactivations (day +33, +47, +90, +132 and +181), that were treated with VGC. Due to GvHD at day 92, high levels of immunosuppressive drugs were administered, which impaired CMV-CTL recovery. By day +201 this patient had 0.056 $IFN\gamma^+CD8^+$ T cells/μL and did not reactivate CMV thereafter.

Patient 15 (D-/R+) had 4 CMV reactivations (day +39, +87, +130 and +186) that were treated with VGC. This patient achieved 0.286 $IFN\gamma^+CD8^+$ T cells/μL by day +300 and did not further reactivate CMV anymore.

Patient 14 (D+/R-) had 4 CMV reactivations that were treated with VGC. This patient received ATG and the lymphocyte count was very low until day +100 (less than 200 lymphocytes/μL). Then, the patient started to recover the lymphocyte function and after the last CMV reactivation on day +159, functional CD8+ T cells were detected upon *in vitro* stimulation (1.036 $IFN\gamma^+CD8^+$ T cells/μL).

2.4.3 Functional CMV-specific $CD8^+$ T-cell recovery patterns

In this case, we have been able to describe three different patterns in our cohort of patients. As it was observed with multimer technology for the monitoring of CMV-specific immune reconstitution, two of the three patterns are classified according to functional recovery and antiviral treatment of the recipients; however, a third pattern has been described when analysing *in vitro* IFNγ production by $CD8^+$ T cells upon stimulation with CMV-$pp65_{495\text{-}503}$ peptide.

This new pattern is formed by a group of patients that never experienced CMV reactivation during the year follow up after allo-HSCT (n=6). Despite finding no differences in the number of virus-specific cells when multimer technology was used at early time points after transplantation (day +30), we have observed that those cells were functional, as measured by *in vitro* IFNγ production whereas this did not occur in patients with reactivation. Therefore, they may protect against CMV reactivation. Median number of IFNγ production levels all over the year was 0.630 $IFN\gamma^+CD8^+$ T-cells/μL (0.094-13.542) (Figure 26A).

The second and most frequent pattern is formed by a group of patients that experienced an increase on the *in vitro* IFNγ production levels by $CD8^+$ T cells after CMV reactivation and therefore requiring short antiviral treatment (n=10). The median number of IFNγ production levels during the year follow up was 2.590 $IFN\gamma^+CD8^+$ T-cells/μL (0.000-6.157) (Figure 26B).

The third pattern described was formed by patients who did not have an increase on IFNγ levels upon *in vitro* stimulation after CMV reactivation episodes and therefore requiring prolonged time of antiviral therapy (n=3) (Figure 26C and D). These patients had no functional $CD8^+$ T cells until day 200 (median of 0.050 $IFN\gamma^+CD8^+$ T-cells/μL (0.000-0.661)) and experienced CMV reactivations after 3 months post-transplant.

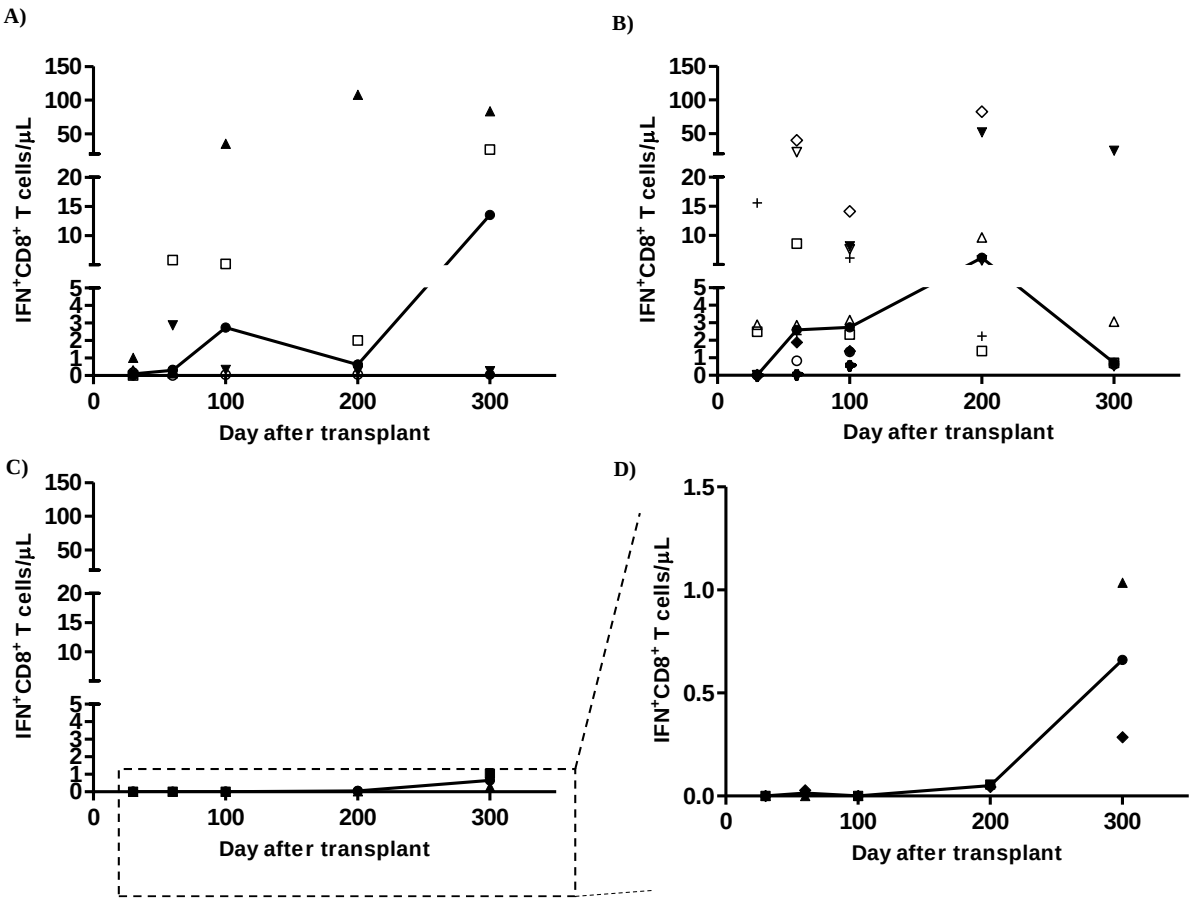

Figure 26. . Different patterns observed in our cohort of individuals. Patients with early functional CMV immune reconstitution in the absence of CMV reactivation (n=6) (A). Patients that had an increase on *in vitro* IFNγ levels after CMV reactivation and required short times of antiviral treatment (n=10) (B) and patients with no *in vitro* IFNγ production levels after CMV reactivation requiring prolonged cumulative antiviral therapy (n=3) (C and D). The black line indicates the median of CMV-CTL levels at days +30, +60, +100, +200 and +300 post-transplant

These results obtained after monitoring CMV-specific immune reconstitution by measuring *in vitro* IFNγ production upon stimulation with CMV-pp65$_{495-503}$ peptide have led us to explain the observations that we had with multimer technique. It is necessary to have not only CMV-specific CD8$^+$ T cells detected by multimer technology but also functional T cells (detected by *in vitro* IFNγ production).

2.5 CMV-CTL levels and functional activity

The previous methods have allowed us to monitor CMV-specific CD8$^+$ T cells recovery by identifying these cells with multimer technology or by measuring their function after stimulation with the specific peptide. The peptide CMV-pp65$_{495-503}$ has HLA-A*02:01 restriction and it is therefore supposed that only CMV-specific CD8$^+$ T cells with that restriction will recognize and respond to the antigen. However, it is also possible to monitor CMV-specific immune reconstitution by measuring cytokines-produced upon stimulation with a specific peptide by CMV-specific T cells that are stained with multimer.

A new technique has recently been described in order to monitor functional activity of CMV-specific CD8$^+$ T cells detected by multimer technique. In our study, we have been able to incorporate this γ-capture-based kit only in 9 of the 25 patients to monitor the *in vitro* IFNγ produced by multimer$^+$CD8$^+$ T cells upon stimulation with CMV-pp65$_{495-503}$ antigen.

Firstly, we wanted to evaluate and to compare if the number of multimer positive cells detected with both processes was the same (direct staining with multimer and multimer detected in the γ-capture process). Due to the low number of processed samples, we have grouped all measurements (n=33) for the analysis. According to what was expected, a strong correlation is observed between both of them ($r_{Spearman}$ = 0.940, $p<0.001$) suggesting that the number of multimer positive cells is maintained after *in vitro* stimulation with the antigen (Figure 27).

Similar to multimer analysis, we wanted to compare if the *in vitro* IFNγ production detected with both methodologies, extracellular (γ-capture) and intracellular (ICS) IFNγ staining upon CMV-pp65$_{495-503}$ peptide stimulation, was the same. However, in this case the IFNγ detected by intracellular staining provided significant higher values than the IFNγ detected by the extracellular staining ($p = 0.002$) (Figure 28A and B). The

correlation observed between both of them is not as good as that obtained with multimer technology ($r_{Spearman}$=0.601, p<0.001, Figure 28C).

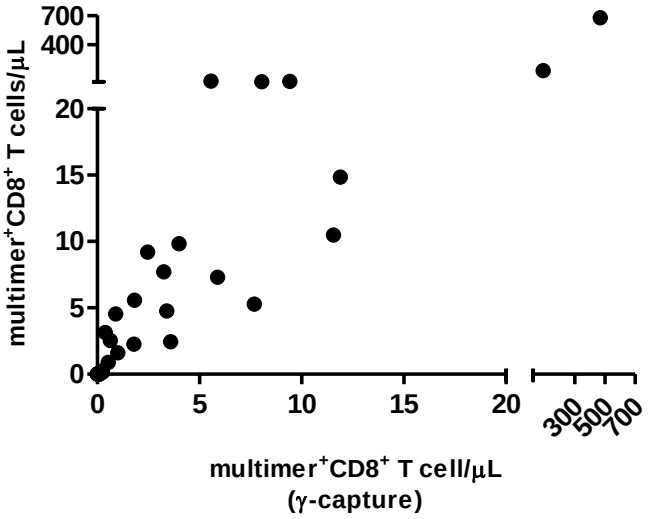

Figure 27. Correlation of multimer positive $CD8^+$ T cells detected with the normal protocol of CMV-specific $CD8^+$ T cells detection (*y*-axis) and the protocol used for the γ-capture (*x*-axis).

Furthermore, when comparing the *in vitro* IFNγ production levels by $CD8^+$ T cells with both techniques, higher IFNγ levels were observed when analysing the intracellular staining at +60 days following transplant compared against extracellular staining (median of 1.194 $IFN\gamma^+CD8^+$ T cells/µL (0.000-40.015) and 0.010 $IFN\gamma^+CD8^+$ T cells/µL (0.000-38.743) respectively; p=0.069).

We studied the IFNγ production levels by the γ-capture technique at day +30 and +60 by multimer positive cells in patients without (n=4) and with (n=5) CMV reactivation to see if we could find any difference to explain the above observation. In this case, two patients of the 9 analysed reactivated CMV during the first month following transplant and they were not considered for the analysis at 30 days after allo-HSCT but they were included at 60 days post-transplantation analysis. No differences were found between patients without and with CMV-DNAemia neither at +30 nor +60 days post-transplant, probably due to the low number of samples analysed at each time point (Table 22).

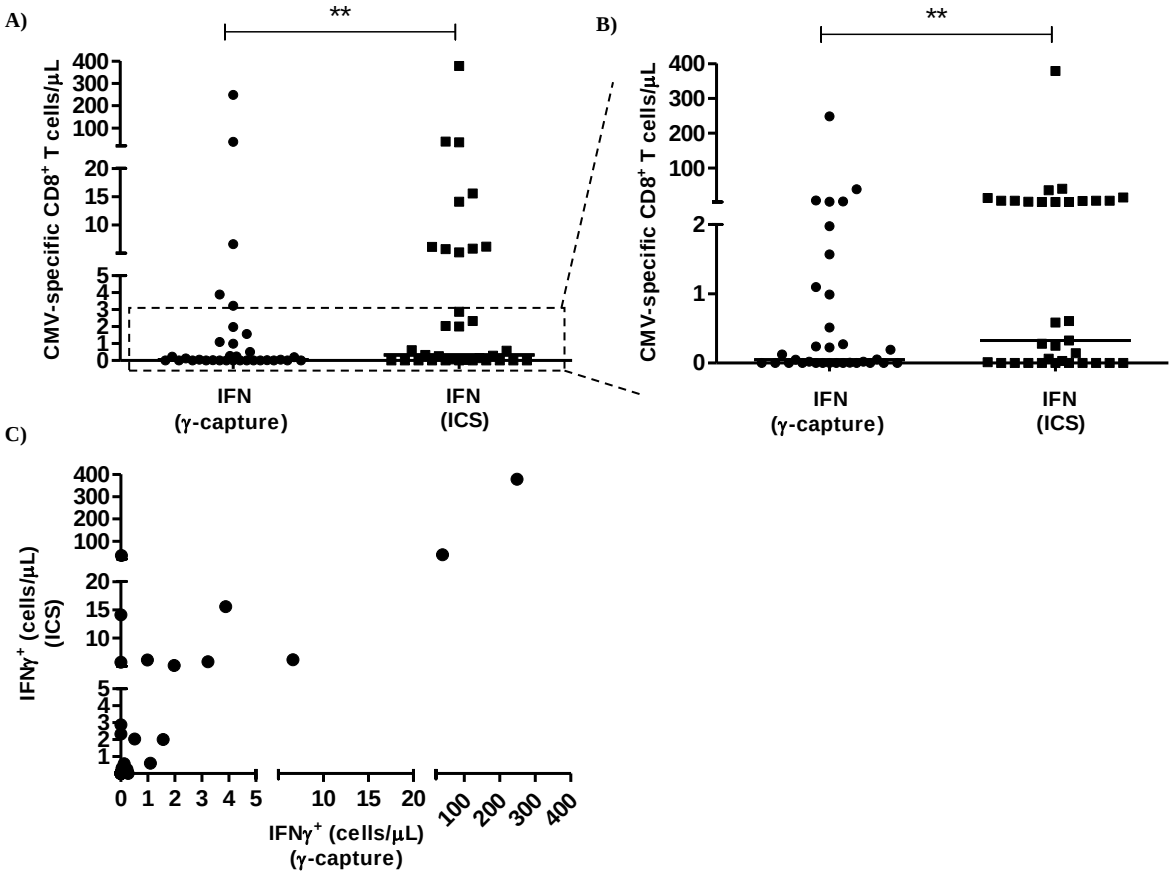

Figure 28. Extracellular and intracellular IFNγ levels detected with both methodologies (A and B) and correlation of IFNγ positive $CD8^+$ T cells detected with intracellular (*y*-axis) and extracellular staining (*x*-axis) (C). **p<0.01.

Table 22. Median of *in vitro* IFNγ production levels by multimer positive cells after stimulation with $CMVpp65_{495\text{-}503}$ antigen in patients without and with CMV reactivation at +30 and +60 days after allo-HSCT.

			Median IFNγ production by multimer positive cells	**Range**
Day +30	**CMV reactivation**	no	0.050	0.000-0.272
		yes	0.000	0.000-0.004
Day +60	**CMV reactivation**	no	0.120	0.000-3.223
		yes	0.010	0.000-38.743

This technology could be a useful tool for the monitoring of CMV-specific $CD8^+$ T-cell immune recovery and its functionality. However, future experiments with higher number of patients would be necessary to arrive to some conclusions.

B Phenotypic characterisation of Cytomegalovirus-specific CD4$^+$ T cells

The presence of CMV-specific CD4$^+$ T cells has been shown through *in vitro* culture. However their phenotype and functional activity are less understood compared to CD8$^+$ T cells and it has been suggested to be altered during culture. In this study, we are going to characterise CMV-specific CD4$^+$ T cells by the direct *ex vivo* staining with tetramer technology in healthy individuals prior to its implementation in the monitoring of allo-HSCT recipients

1 Specificity CMV-specific CD4$^+$ T-cell clones

In order to study CMV-specific CD4$^+$ T cells as explained below (section B3), it was necessary to generate CD4$^+$ T-cell clones to test specificity and evaluate the sensitivity of MHC class II tetramers. The 'potential' CMV-specific CD4$^+$ T-cell clones generated were directed against the CMV glycoprotein B (gB)-derived epitope DYSNTHSTRYV, residues: 217-227 in the context of HLA-DRB1*07:01-restricted and CMV pp65-derived epitopes AGILARNLVPMVATV, residues: 489-503 with HLA-DRB3*02:02 restriction, and epitope LLQTGIHVRVSQPSL, residues: 41-55 with HLA-DQB1*06:02 restriction. The specificity of each clone was evaluated by measuring the *in vitro* IFNγ production after stimulation with the appropriate peptide-loaded LCL ($gB_{217-227}$, $pp65_{41-55}$ or $pp65_{489-503}$) and DMSO-loaded as negative control. The following figure shows an example of the DYS-, AGI- and LLQ-specific CD4$^+$ T-cell clones (clone A, clone C and clone E, respectively) and some 'potential' clones that were not CMV-specific as they did not respond to their cognate CMV antigen (clone B, clone D and clone F) after stimulation with DYS, AGI or LLQ, respectively (Figure 29).

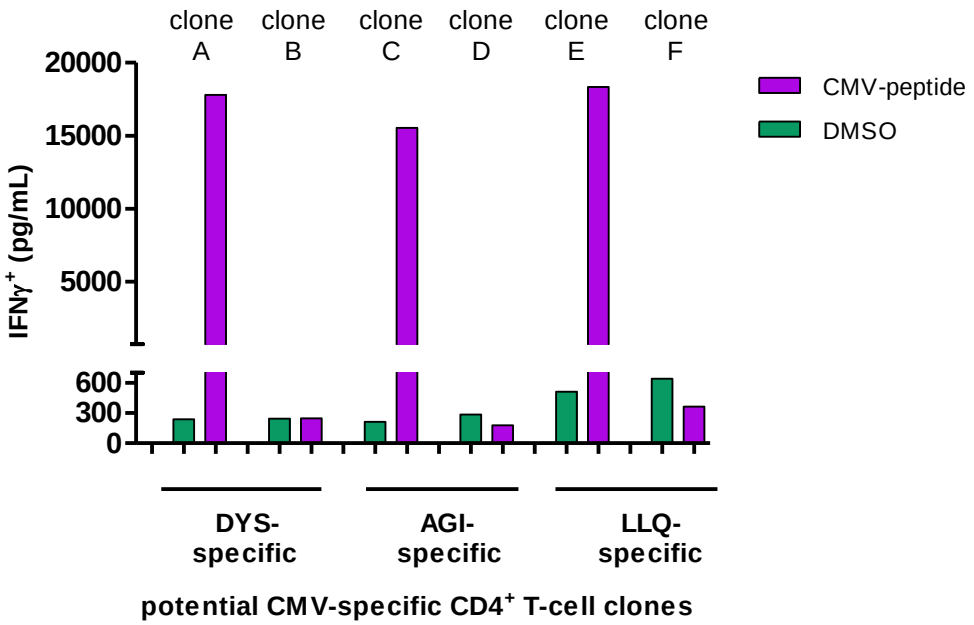

Figure 29. Representative examples of *in vitro* IFNγ$^+$ production by 'potential' CMV-specific CD4$^+$ T-cell clones after stimulation with either DYS-, AGI- and LLQ-peptides or DMSO as negative control.

2 Validation of MHC class II tetramers

As described in Materials and Methods (section B1), a total of 55 CMV seropositive healthy volunteers were recruited into the study in order to characterise the phenotype of CMV-specific CD4$^+$ T cells with the use of MHC class II tetramers. The donor cohort was grouped according to their age into young (18-40 years), middle aged (41-60 years) and older adults (over 60 years).

We have used three MHC class-II tetrameric complexes that were directed against the CMV $gB_{217-227}$ (DYS) in the context of HLA-DRB1*07:01 (DR7) restricted, or against the CMV $pp65_{489-503}$ (AGI) with HLA-DRB3*02:02 (DR52b) restriction and CMV $pp65_{41-55}$ (LLQ) HLA-DQB1*06:02 (DQ6) restriction.

Before performing the phenotypic analyses of CMV-specific CD4$^+$ T cells, the specificity of each tetramer was evaluated.

2.1 Specificity and sensitivity of MHC class II tetramers

Initially, the specificity of each tetramer was confirmed by screening against epitope-specific $CD4^+$ T-cell clones or PBMCs from an HLA-matched CMV-seronegative donor. All three tetramers are highly specific as the background levels observed when staining PBMCs from a CMV-seronegative donor were very low (less than 0.06%) whereas more than 95% positive cells were observed when staining the CMV-specific $CD4^+$ T-cell clone that recognizes the tetramer's cognate MHC II-peptide complex.

Similar results for each epitope were obtained when analysing decreasing amounts of $CD4^+$ T-cell clones specific for DYS-, AGI- or LLQ-epitopes in a mixture with PBMCs from a CMV-seronegative donor with the corresponding HLA restriction (DR7, DR52b or DQ6, respectively) (Table 23 and Figure 30). For that purpose, the $CD4^+$ T-cell clone was diluted with PBMCs from the CMV-seronegative donor to have 5%-1%-0.5%-0.25%-0.10% of peptide-specific $CD4^+$ T cells. A strong correlation was obtained with each tetramer with the predicted positive (Table 23). These results showed that each tetramer was able to detect low frequencies of epitope-specific T cells (as low as 0.1% CMV-specific $CD4^+$ T cells).

Table 23. Percentage of the DYS-, AGI- and LLQ-specific $CD4^+$ T-cell clones after dilution with PBMCs from a CMV-seronegative donor with the same HLA restriction.

Tetramer	Percentage of the $CD4^+$ T-cell clone (%)						Negative donor	ICC	*p value*
Theoric	100.00	5.00	1.00	0.50	0.25	0.10	0.00		
DYS	97.70	6.93	1.13	0.57	0.27	0.14	0.01	1.000	*<0.001*
AGI	95.77	3.57	0.80	0.38	0.21	0.11	0.05	0.999	*<0.001*
LLQ	98.18	13.72	2.94	1.13	0.60	0.35	0.06	0.996	*<0.001*

ICC = Inctraclass Correlation Coefficient

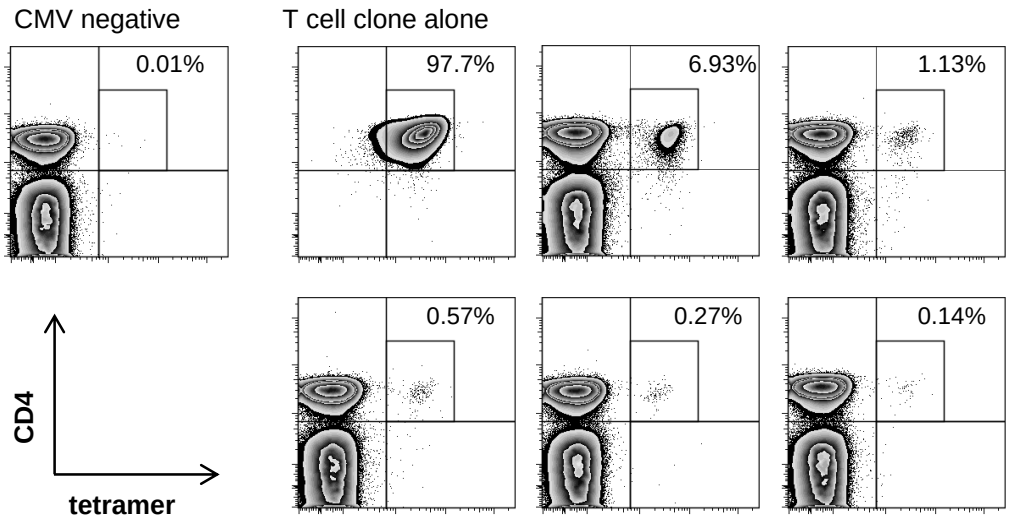

Figure 30. Representative dot plots of decreasing amounts of DYS-specific $CD4^+$ T-cell clone with PBMCs from a CMV-seronegative HLA-DR7 positive donor.

Once the specificity and sensitivity of each class II tetramer was confirmed with the use of T-cell clones, the validation of each tetramer was performed by using PBMCs from CMV-seropositive healthy donors with HLA DR7, DR52b and DQ6. PBMCs were stained with DYS, AGI or LLQ class II tetramers and CD3 and CD4 surface markers. PBMCs from 3 CMV-seronegative donors with HLA DR7, DR52b and DQ6 were also stained as negative controls. As it was observed with clones, the specificity of each MHC class II tetramer was confirmed with PBMCs from healthy donors and they can be used to detect CMV-specific $CD4^+$ T cells directly *ex vivo* (Figure 31). The range of detection of CMV-specific $CD4^+$ T cells was different between different epitopes in our cohort of donors. DYS-specific $CD4^+$ T cells ranged from 0.10 to 11.75% of the $CD4^+$ T-cell population; AGI-specific $CD4^+$ T cells ranged from 0.07 to 2.97% of total $CD4^+$ T cells and LLQ-specific $CD4^+$ T cells were between 0.10 to 24.02% of $CD4^+$ T cells.

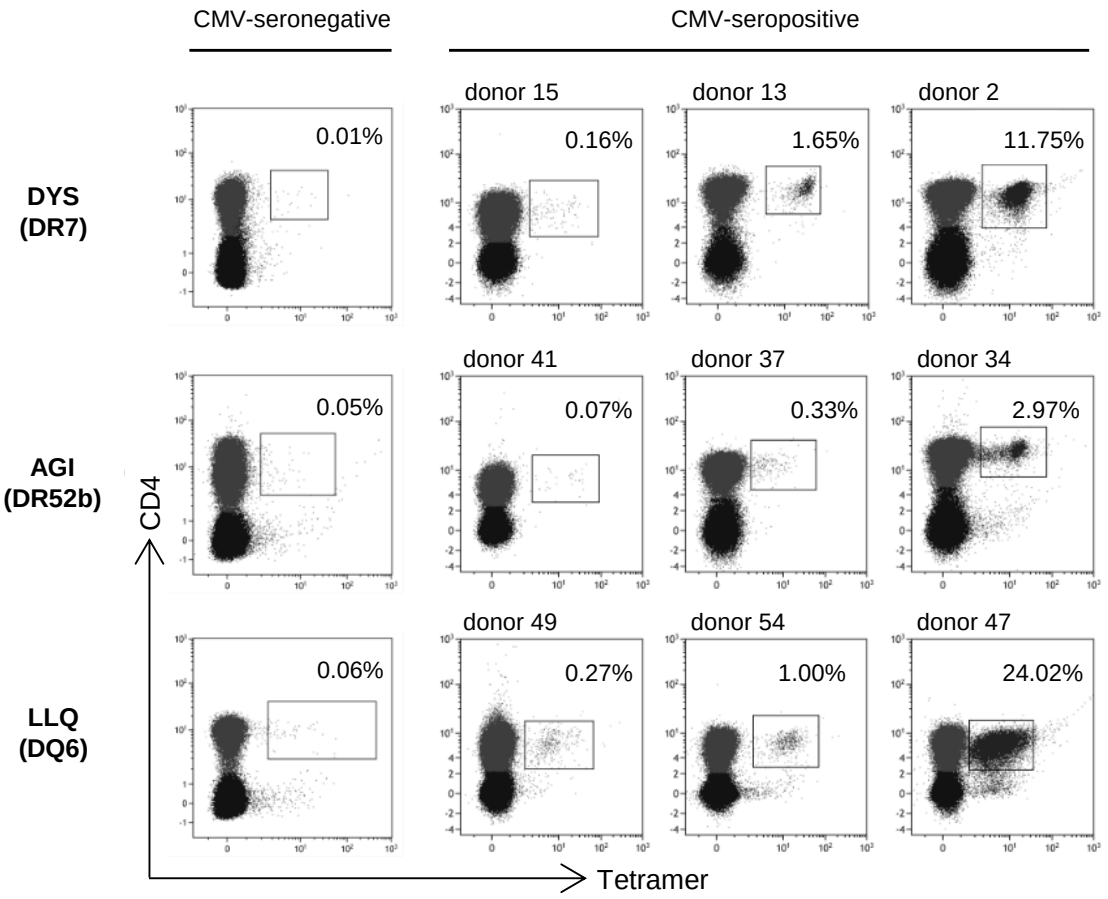

Figure 31. Representative dot plots of 3 different CMV-seropositive donors per HLA restriction (DR7, DR52b and DQ6). CMV-seronegative healthy donors were used as negative control.

3 Phenotypic characterisation of CMV-specific CD4$^+$ T cells

According to previous *in vitro* studies CMV-specific CD4$^+$ T cells showed an effector memory phenotype in healthy individuals. However, we were interested in characterising their phenotype by direct staining *ex vivo* with class II tetramers without prior peptide stimulation.

Firstly, we grouped donors according to their ages as young (n=16), middle aged (n=26) and older adults (n=13) and, subsequently, PBMCs from those donors were stained with the appropriate class II tetramer. We have observed that the frequency of CMV-specific CD4$^+$ T-cell levels did not increase with age (Table 24 and Figure 32).

Table 24. Median frequencies of CMV-specific $CD4^+$ T cells found in the different cohort of individuals.

	Median CMV-specific $CD4^+$ T cells (%)	**range**
Young (n=16)	0.78	0.10-7.62
Middle aged (n=26)	0.28	0.07-2.97
Older adults (n=13)	0.72	0.10-24.02

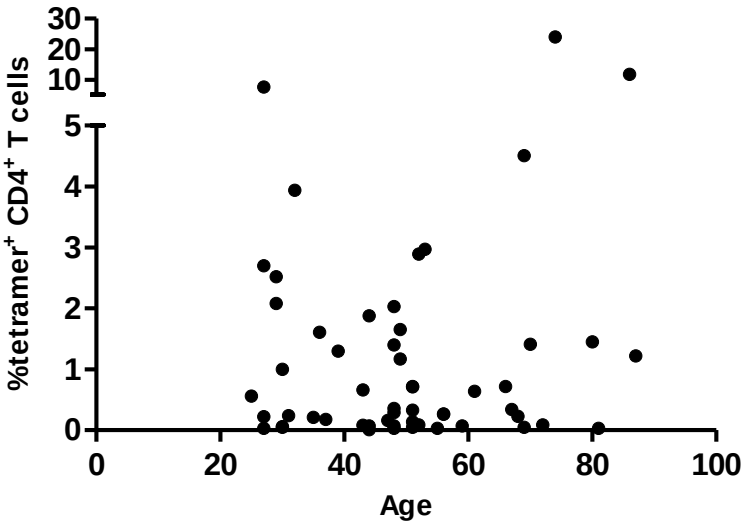

Figure 32. Relation between $tetramer^+CD4^+$ T cells and age.

By looking at the different epitopes, a total of 29 DYS- (DR7); 17 AGI- (DR52b) and 10 LLQ- (DQ6) positive donors (n = 55) were stained with MHC class II tetramer in order to characterise the phenotype of CMV-specific $CD4^+$ T cells directly *ex vivo*.

The majority of DYS- (DR7), AGI- (DR52b) and LLQ- (DQ6) specific $CD4^+$ T cells expressed high levels of CD45RO with a median of 97.22% (29.33–100) and lacked the expression of CCR7 showing an effector memory (EM) phenotype (Figure 33A). CD45RA expression was low for AGI- and LLQ-specific T cells (median 3.45% $CD45RA^+$ (0–23.53) and 6.66% $CD45RA^+$ (0-24.24), respectively) but higher values were found on some DYS-specific T cells (median of 10.87% $CD45RA^+$ (0–82.04)) (Figure 33B), therefore showing a revertant memory phenotype (EMRA). The EM phenotype ($CD45RA^-CCR7^-$) was not related to age and it was observed in all three donors groups (Figure 33C).

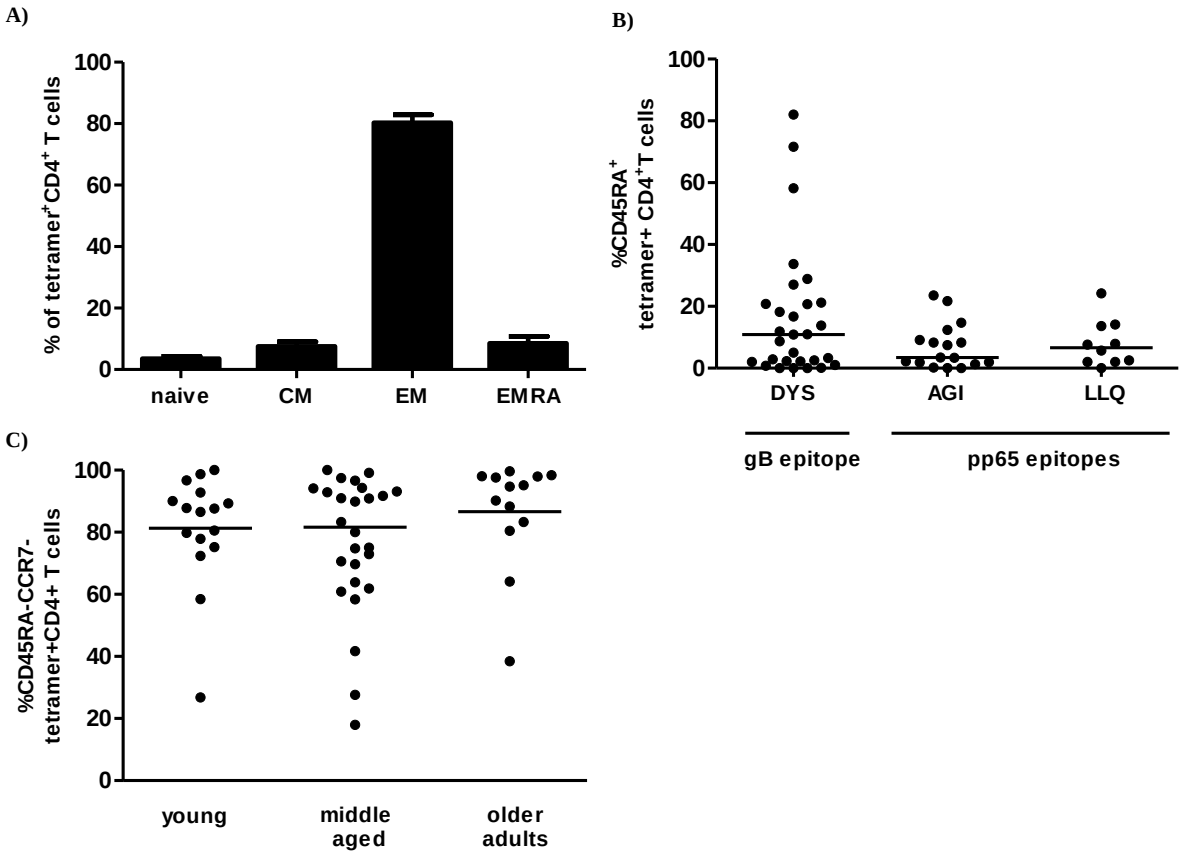

Figure 33. Effector memory phenotype as defined by expression of CCR7 and CD45RA presented by CMV-specific $CD4^+$ T cells (A). Expression levels of CD45RA marker by DYS- (DR7), AGI- (DR52b) and LLQ- (DQ6) specific $CD4^+$ T cells (B). Levels of $CD45RA^-CCR7^-$ expression on CMV-specific $CD4^+$ T cells in young, middle aged and older adults (C).

The expression of the co-stimulatory molecules CD28 and CD27 as well as CD57 allows to identify different stages of differentiation status that T cells present. In that sense, when looking at the expression of these markers on CMV-specific $CD4^+$ T cells we did not observe a significant decrease of CD27 and CD28 with age, although there was lower expression in older adults. CD57 expression confirmed these observations as there was a slightly increased with age (p=0.059) (Table 25).

Table 25. Median frequencies of CD28, CD27 and CD57 expression on young, middle aged and older adults.

	Median %CD28	**range**	**Median %CD27**	**range**	**Median %CD57**	**range**
Young (n=16)	62.09	9.76-100.00	14.67	0.00-99.01	17.50	0.00-65.10
Middle aged (n=26)	70.73	1.89-100.00	14.80	1.35-82.76	23.44	0.00-84.71
Older adults (n=13)	33.33	0.82-92.31	7.37	0.24-46.15	41.30	0.00-79.56

By contrast, significant differences were found in the expression of these markers according to the three different epitopes (gB (DYS) and pp65 (AGI and LLQ) epitopes). The expression of the co-stimulatory molecule CD28 was very similar on T cells specific for both pp65 epitopes (AGI and LLQ) but significantly lower on gB (DYS)-specific $CD4^+$ T cells with a median of 31.07% $CD28^+$ (0.82-95.62) for DYS compared to 75.00% $CD28^+$ (15.31-100) for pp65 epitopes ($p<0.001$) (Figure 34A and Table 26). Expression of CD28 slightly decreased with age only on DYS-specific $CD4^+$ T cells ($r_{Spearman}$= -0.3582; p=0.056) (Figure 34B).

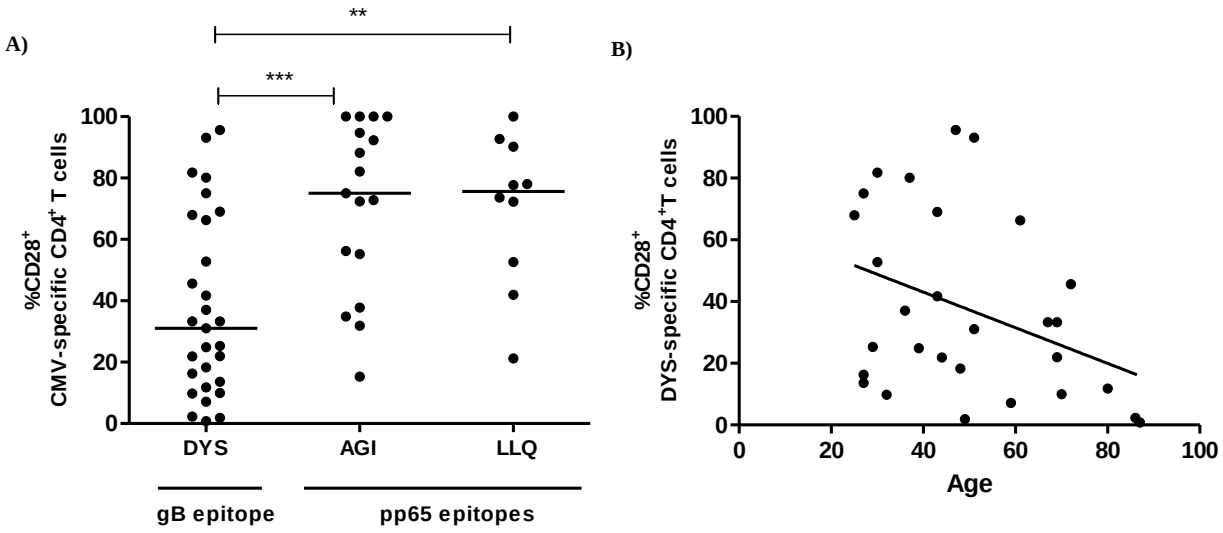

Figure 34. Expression of co-stimulatory molecule CD28 on DYS-, AGI- and LLQ-specific $CD4^+$ T cells (A). CD28 expression decreases with age on DYS-specific $CD4^+$ T cells ($r_{Spearman}$=-0.3582; p=0.056) (B). **<0.01, ***<0.001.

Table 26. Median frequencies of CD28, CD27 and CD57 expression on DYS, AGI and LLQ specific $CD4^+$ T cells.

Median % (range)	AGI (n=17)	*p-value* (DYS *vs* AGI)	DYS (n=29)	*p-value* (DYS *vs* LLQ)	LLQ (n=10)
CD28	75.00 (15.31-100.00)	*<0.001*	31.07 (0.82-95.62)	*0.006*	75.66 (21.21-100.00)
CD27	28.03 (1.38-70.59)	*0.010*	7.37 (0.00-99.01)	*0.135*	12.87 (5.35-62.93)
CD57	12.78 (0.00-81.00)	*<0.001*	41.30 (0.00-84.71)	*0.010*	20.16 (0.00-51.52)

CD57 expression, a characteristic marker of highly differentiated T cells, showed marked variation in CMV-specific $CD4^+$ T cells with an increase of 3 fold on DYS-specific $CD4^+$

T cells compared with AGI-specific $CD4^+$ T cells ($p<0.001$) and 2 fold with respect to LLQ-specific $CD4^+$ T cells ($p=0.012$) (Figure 35A) (Table 26). CD57 expression significantly increased with age on DYS-specific $CD4^+$ T cells ($r_{Spearman}=0.508$; $p=0.005$) (Figure 35B).

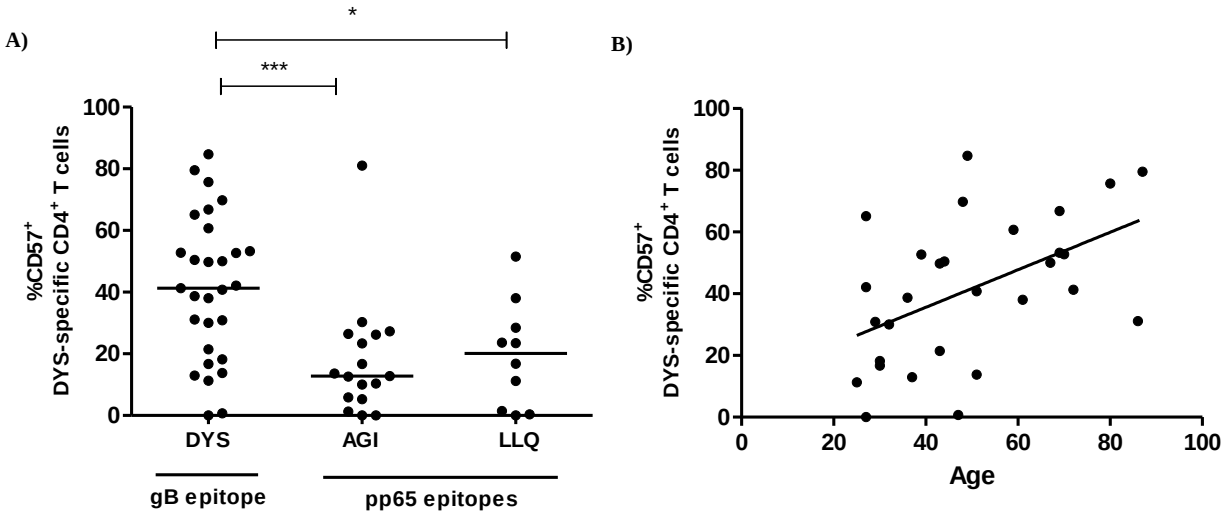

Figure 35. Expression of CD57 molecule on DYS-, AGI- and LLQ-specific $CD4^+$ T cells (A). Expression of CD57 molecule on DYS-specific $CD4^+$ T cells significantly increases with age ($r_{Spearman}=0.508$; $p=0.005$) (B). *<0.05, ***<0.001.

The loss of the co-stimulatory receptor CD27 expression seems to be associated with CMV infection (179). In our cohort of individuals we have observed that CMV-specific $CD4^+$ T cells presented lower levels of CD27 expression than CD28 with a median of 12.80% $CD27^+$ (0–99.01%) and 55.53% $CD28^+$ (0.82–100%), respectively, but no differences were found related to age.

However, when comparing $CD4^+$ T cells specific for different epitopes, larger differences were found. For both pp65-derived epitopes, AGI and LLQ, the majority of specific T cells lacked the expression of the CD27 co-stimulatory molecule but expressed CD28 showing marked differences with the gB-derived epitope as the majority of these cells lacked CD28 co-stimulatory molecule with a median of 69.42% DYS-specific $CD4^+CD27^-CD28^-$ T cells (0.49-99.18%) compared to 27.04% pp65-epitope-specific $CD4^+CD27^-CD28^-$ T cells (0.00-78.97%) ($p<0.001$). These observations suggest an end-stage of differentiation of DYS-specific $CD4^+$ T cells compared to AGI- and LLQ-specific $CD4^+$ T cells (Figure 36).

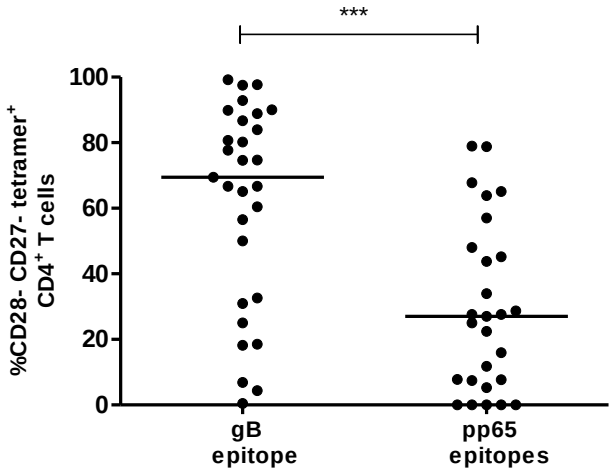

Figure 36. Proportion of CD27-CD28- cells within the multimer positive $CD4^+$ T-cell population according to proteins (gB or pp65). ***p<0.001.

Therefore, CMV-specific $CD4^+$ T cells showed an effector memory phenotype which was not related to donor age, and DYS-specific $CD4^+$ T cells showed a late differentiated phenotype characterised by the loss of expression of co-stimulatory molecules such as CD27 and CD28 and increase expression of the CD57 marker that was related to age.

4 Functionality of CMV-specific $CD4^+$ T cells

4.1 Cytotoxic potential of CMV-specific $CD4^+$ T cells

The cytotoxic potential of CMV-specific $CD4^+$ T cells after *in vitro* stimulation has reported the use of perforin-dependent cytotoxic mechanism rather than the Fas-dependant pathway. However, some authors have suggested that this cytotoxic capacity may be acquired during *in vitro* culture.

For that reason, we have analysed the expression of two cytolytic molecules, granzyme B and perforin, and the expression of Fas ligand (FasL) in our cohort of donors by direct staining with tetramer multimer. FasL expression was very low within CMV-specific $CD4^+$ T cells with a median of 0.45% $FasL^+$ (0.00-12.84%) and it was not related with age suggesting that CMV-specific $CD4^+$ T cells do not use this pathway as mechanism of cytotoxicity.

In general, few $CD4^+$ T cells with granzyme B and perforin containing granules were detected in the circulation of most healthy individuals (median 7.86% $CD4^+granzymeB^+$ (0.66-75.62) and 5.26% $CD4^+perforin^+$ (0.27-36.63)) but significant differences were found when comparing these with CMV-specific $CD4^+$ T cells. CMV-specific T cells expressed much higher levels of both molecules (median 73.91% (0.00-97.18%) and 45.35% (0.00-93.60), granzyme B and perforin levels respectively, $p<0.001$) (Figure 37).

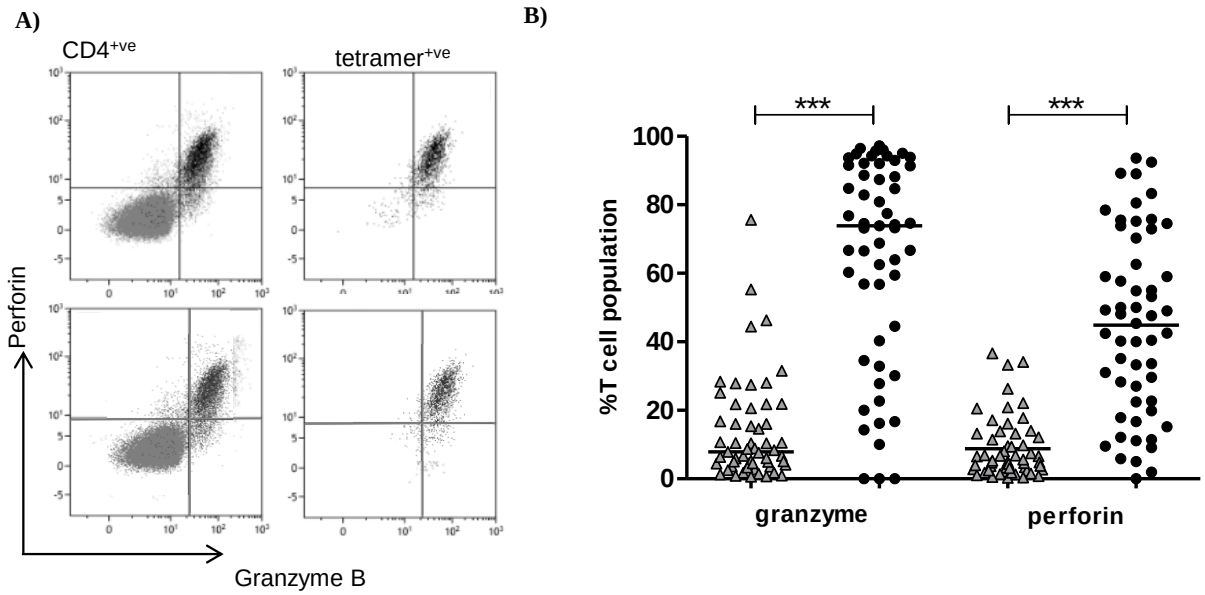

Figure 37. Representative dot plots showing of expression of cytotoxic molecules granzyme B and perforin in $CD4^+$ T cells and tetramer positive cells (A). Expression levels of granzyme B and peforin in $CD4^+$ T cells (grey triangles) and CMV-specific $CD4^+$ T cells (black circles) (B). ***$p<0.001$.

Further differences were observed between gB- and pp65-specific T cells with an increase of 2.7 fold of perforin expression on DYS-specific $CD4^+$ T cells (DR7-restricted) compared to AGI-specific $CD4^+$ T cells (DR52b-restricted) ($p<0.001$) and 1.4 fold increase compared to LLQ (DQ6-restricted) ($p=0.04$) (Figure 38A) (Table 27). Equally to perforin expression, DYS-specific $CD4^+$ T cells were found to have greater granzyme B levels (median 84.77% (16.67-97.18)) than AGI- and LLQ-specific $CD4^+$ T cells (median 40.27% (0.00-96.48); $p<0.001$ and 67.68% (0.00-91.52); $p=0.04$, respectively) (Figure 38B). A positive correlation was observed between granzyme B and perforin values ($r_{Spearman} = 0.832$; $p<0.001$) (Figure 39).

Table 27. Median frequencies of perforin and granzyme B expression in DYS-, AGI- and LLQ-specific $CD4^+$ T cells.

Median % (range)	AGI (n=17)	*p-value* (DYS *vs* AGI)	DYS (n=28)	*p-value* (DYS *vs* LLQ)	LLQ (n=10)
perforin	22.73 (0.00-74.52)	*<0.001*	60.81 (9.09-93.60)	*0.04*	42.49 (588-59.07)
granzyme B	40.27 (0.00-96.48)	*<0.001*	84.77 (16.67-97.18)	*0.04*	67.68 (0.00-91.52)

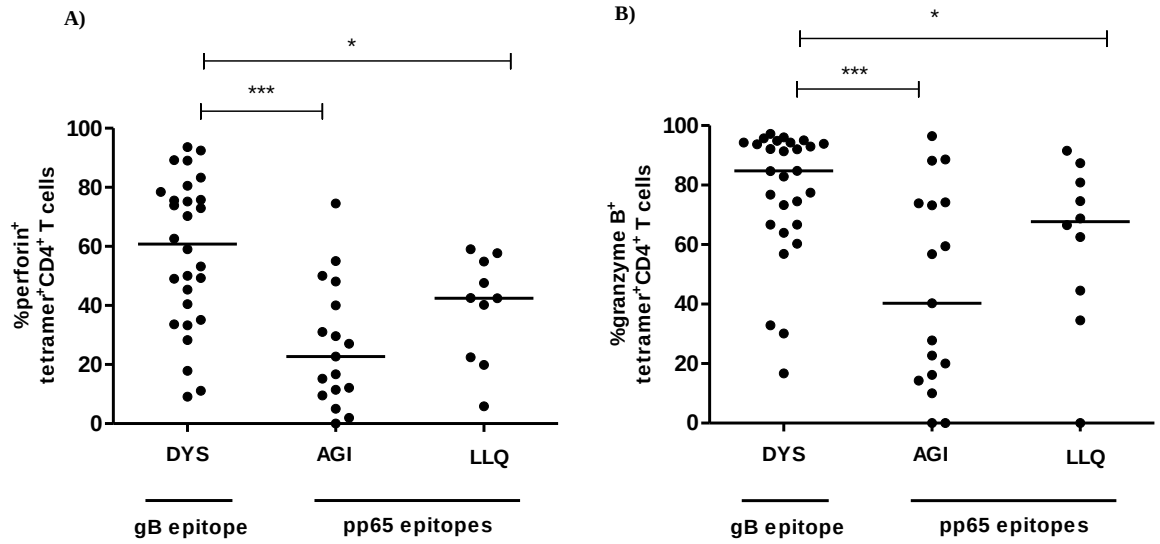

Figure 38. Percentage of expression levels of perforin (A) and granzyme B (B) in DYS-, AGI- and LLQ-specific $CD4^+$ T cells. *p<0.05, ***p<0.001.

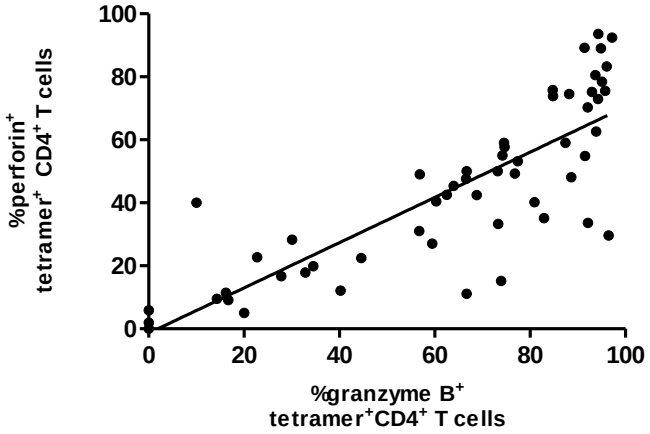

Figure 39. Correlation between perforin and granzyme B expression in CMV-specific $CD4^+$ T cells ($r_{Spearman}$=0.832; p<0.001).

The expression of NKG2D, a killer lectin-like receptor normally displayed by NK cells as well as TcR$\alpha\beta^+$ cells and TcR$\gamma\delta^+$ $CD8^+$ T lymphocytes, has also been described on a particular subset of $CD4^+$ T cells in patients with cancer, chronic autoimmune diseases or persistent infection, and after *in vitro* stimulation its expression increases (180, 181). In that sense, we have evaluated the expression of NKG2D on CMV-specific $CD4^+$ T cells. There was a notable increase of NKG2D expression on CMV-specific $CD4^+$ T cells compared to the overall $CD4^+$ T cells, suggesting that CMV-specific $CD4^+$ T cells were more differentiated (median 22.98% (0.00-73.78) *vs.* 1.57% (0.94-10.40), $p<0.001$, respectively) (Figure 40A). No differences were found in the group of individuals based on age with a median of 21.32% (11.56-54.05), 30.96% (0.00-50.00) and 20.16% (0.93-73.78) for young, middle aged and older adults respectively (Figure 40B). NKG2D expression was pretty similar in gB- and pp65-specific $CD4^+$ T cells and no differences were found (median of 21.33% (0.00-73.78) for DYS-specific $CD4^+$ T cell, 38.33% (3.80-54.05) and 12.78% (5.22-39.07) for AGI- and LLQ-specific $CD4^+$ T cells, respectively) (Figure 40C).

4.2 Analysis of regulatory function of CMV-specific $CD4^+$ T cells

A subset of $CD4^+$ T cells is known to act as regulatory T cells (T regs) by supressing T-cell responses against self-antigens. $CD4^+$ T regs are characterised by expressing the surface marker CD25, a marker of T-cell activation that also mediates suppressor T-cell function (182), the intracellular FoxP3 transcriptor factor but have low or no CD127 expression. We have evaluated if CMV-specific $CD4^+$ T cells have regulatory function by directly *ex vivo* staining for these markers in combination with tetramer multimer. However, no presence of T regs was observed within the population of CMV-specific $CD4^+$ T cells. No $FoxP3^+$ cells were detected among the specific cells compared to overall $CD4^+$ T cells which on average contained 2.30% (0.20-6.46) of $FoxP3^+$ cells ($p<0.001$) (Figure 41).

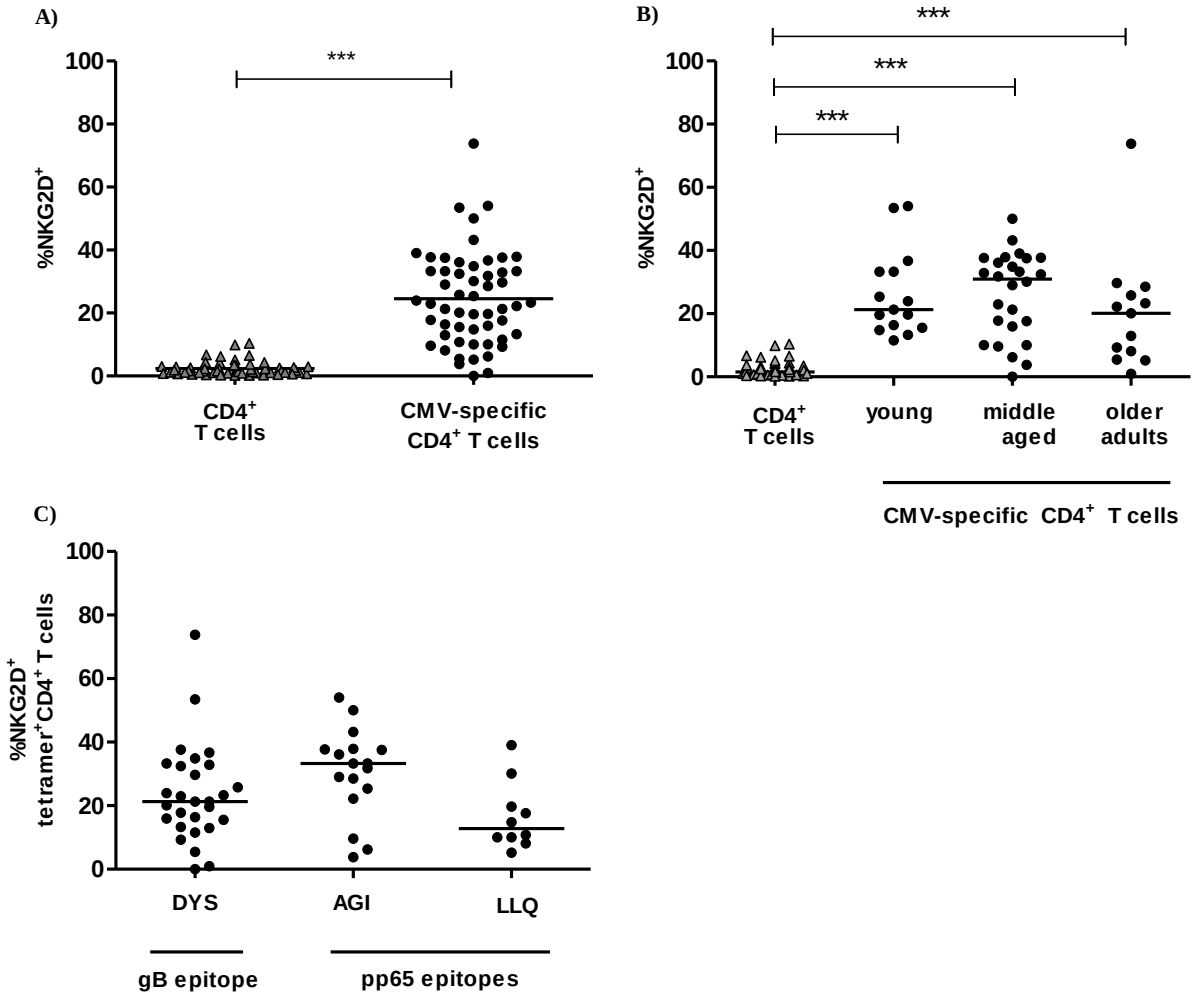

Figure 40. Expression levels of NKG2D marker on total $CD4^+$ T cells and CMV-specific $CD4^+$ T cells (A). Expression of NKG2D on $CD4^+$ T cells and CMV-specific $CD4^+$ T cells in the three groups of individuals based on age (B). Expression of NKG2D on DYS-, AGI- and LLQ-specific $CD4^+$ T cells (C). ***p<0.001

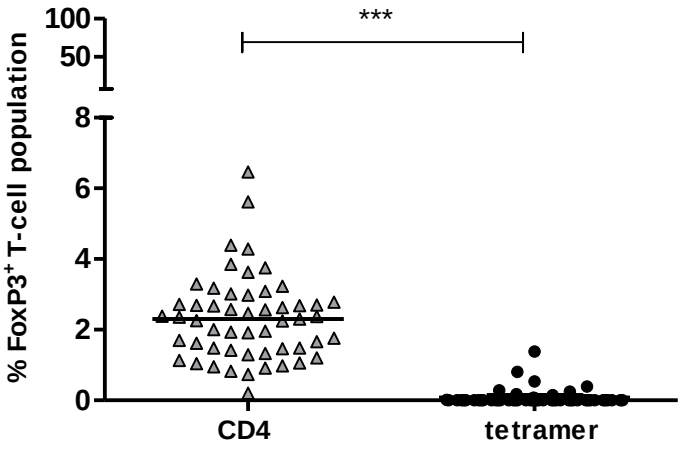

Figure 41. Expression levels of the regulatory marker FoxP3 on total $CD4^+$ T cells and CMV-specific $CD4^+$ T cells. ***p<0.001.

4.3 Functional exhaustion of CMV-specific CD4$^+$ T cells

Functional exhaustion of T cells is induced by continued exposure to high levels of viral antigens and is characterised by partial or complete loss of capacity to produce effector cytokines. There is an increased expression of inhibitory receptors, such as PD-1 and T-cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) and this increase is thought to be associated with late differentiation phenotype characterised by the loss of CD27 and CD28.

In our cohort of individuals no expression of Tim-3 among the CMV-specific CD4$^+$ T cells was observed and it was hardly expressed on overall CD4$^+$ T cells (less than 1.5%). In contrast, the CMV-specific cells expressed the inhibitory molecule PD-1 and there was a marked increase compared to total CD4$^+$ T cells (median 44.93% (5.72-96.97) *vs.* 8.98% (1.60-32.03), respectively; p<0.001) (Figure 42A), indicating that CMV can induce functional impairment of CD4$^+$ T cells. No differences of PD-1 expression were observed with age but there was a significant increase on cells specific for both pp65-derived epitopes when compared to gB-specific T cells (median of 50.65% PD-1 (5.96-96.97) for pp65- and 28.92% PD-1 (5.72-81.82) for gB-specific T cells (p = 0.006)) (Figure 42B).

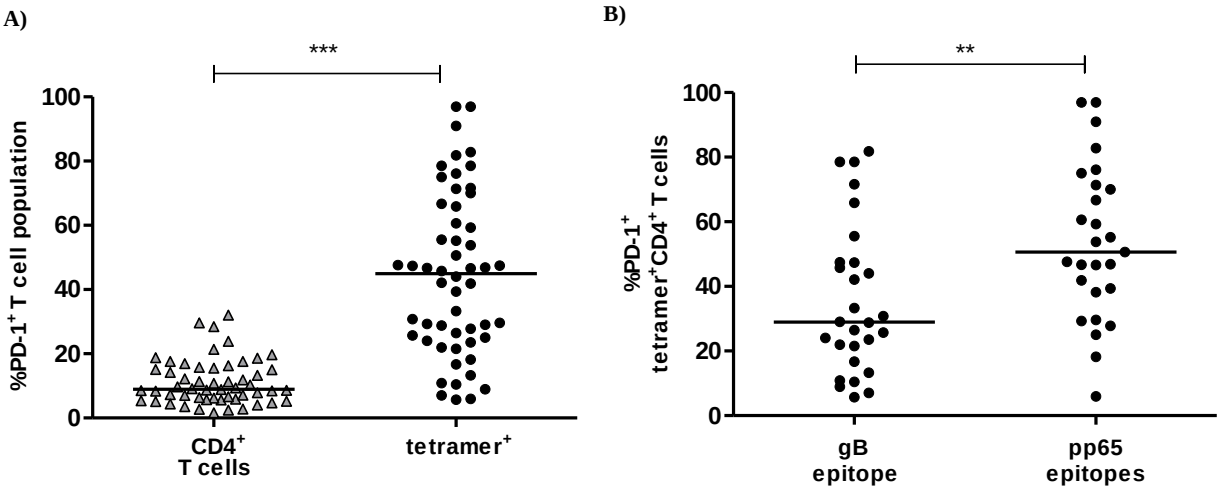

Figure 42. Expression of the inhibitory molecule PD-1 on total CD4$^+$ T cells compared to tetramer positive cells (A) and PD-1 expression on DYS- and AGI&LLQ- (pp65-) specific CD4$^+$ T cells (B). **p<0.01, ***p<0.001.

4.4 Proinflammatory receptor expressed on CMV-specific CD4$^+$ T cells

Latent CMV infection has been related to vascular diseases and immunosenescence (183). During CMV latency, there is a pool of highly differentiated effector T cells that produce inflammatory mediators which can activate endothelial cells that are infected by CMV. These activated endothelial cells produce cytokines such as fractalkine which can draw in immune cells which may be able to induce endothelial damage. The receptor for fractalkine is CX3CR1 and recent studies have shown that it is only expressed on CMV-specific effector CD8$^+$ T (184) and we wanted to know if CMV-specific CD4$^+$ T cells also expressed it.

CX3CR1 was highly expressed on CMV-specific T cells compared to the total CD4$^+$ T-cell pool with no differences with age, showing a median of 84.07% (13.46-98.84) on virus-specific CD4$^+$ T cells and 9.52% (1.16-55.91) on the total CD4$^+$ T-cell population ($p<0.001$) (Figure 43).

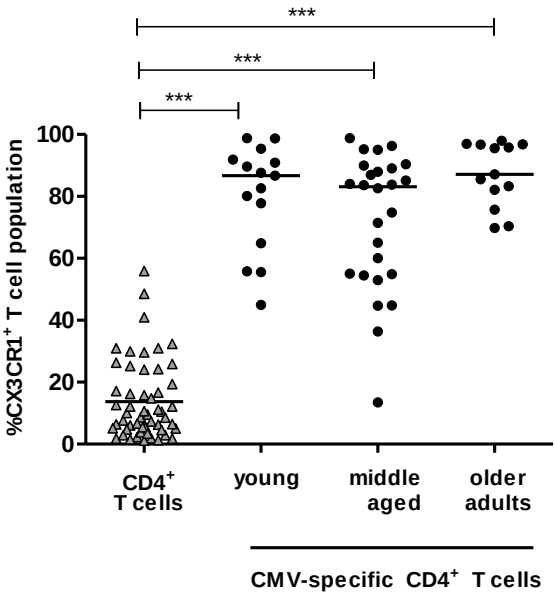

Figure 43. Expression of the fractalkine receptor (CX3CR1) on the total CD4$^+$ T-cell population and CMV-specific CD4$^+$ T cells within the different age groups.***$p<0.001$.

However, when studying the expression of CX3CR1 between different epitope-specific CD4$^+$ T cells, we have observed that there is a significant increase of the expression of this marker on DYS-specific CD4$^+$ T cells compared to pp65 -specific CD4$^+$ T cells

(median of 89.37% (44.70-98.79) and 82.14% (13.46-96.27), respectively; p=0.008) (Figure 44).

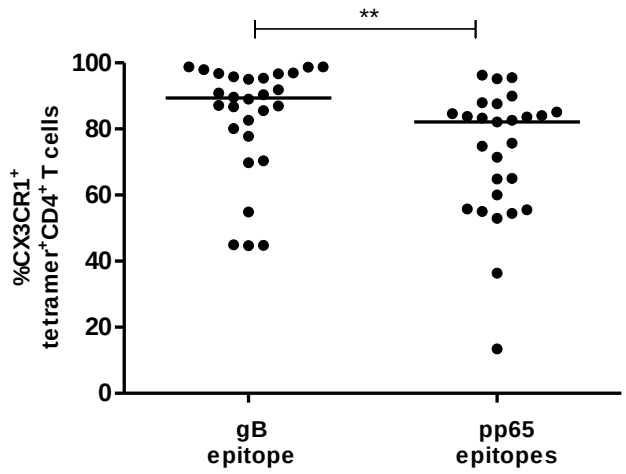

Figure 44. Expression of the fractalkine receptor (CX3CR1) on CMV-specific $CD4^+$ T cells of individuals with cells specific for the gB epitope and both pp65 epitopes.**p<0.01.

The following figure (Figure 45) summarises expression levels of the different surface markers studied on CMV-specific $CD4^+$ T cells of healthy individuals identified by staining with tetramer multimer and the total $CD4^+$ T-cell population. This allows to observe the different phenotypes depending on the epitope-specificity and also the differences that CMV induces on these cells when comparing with the overall $CD4^+$ subset. There is also correlation between the different markers

As a summary, CMV-specific $CD4^+$ T cells lose expression of the co-stimulatory molecule CD27 and acquire cytotoxic function in comparison to the overall $CD4^+$ T cells. CMV-specific $CD4^+CD28^-$ cells expressed higher levels of both granzyme B and perforin cytotoxic molecules. Besides, DYS-specific $CD4^+$ T cells show a highly differentiated phenotype with lower levels of CD28 and increased expression of CD57 surface markers, and have higher cytotoxic potential with higher expression of perforin when compared to pp65-specific T cells.

In conclusion, MHC class II tetramers allow a reliable detection of CMV-specific $CD4^+$ T cells and it has been possible to characterise the phenotype of those cells *ex vivo* in healthy individuals prior to implementing this technology in the monitoring of CMV-specific $CD4^+$ T cells immune recovery following allo-HSCT.

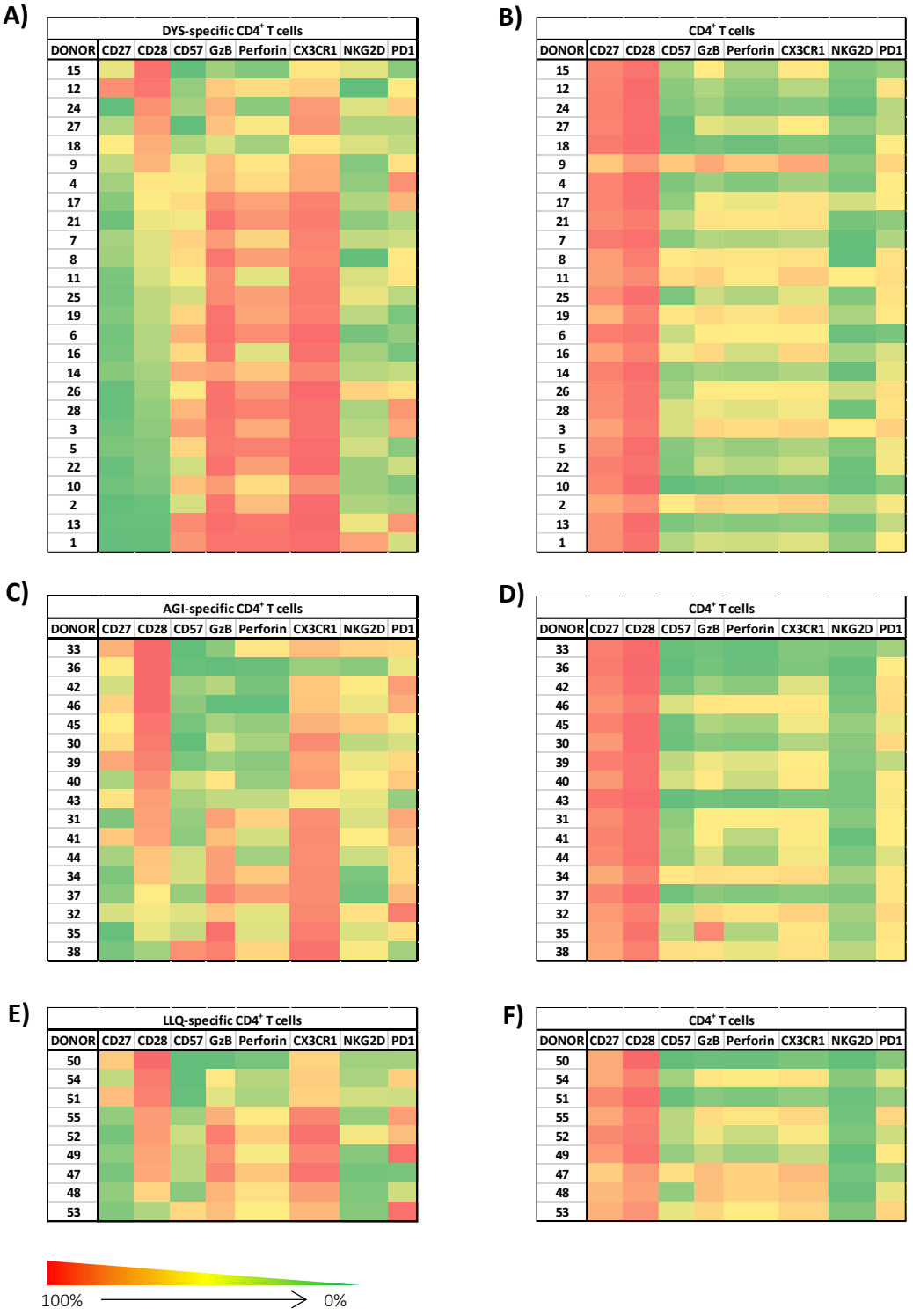

Figure 45. Heatmaps of expression levels of phenotypic markers studied in CMV seropositive healthy donors. Phenotype of CMV-specific CD4$^+$ T cells of DYS-. AGI- and LLQ-specific cells (A, C and E respectively) and phenotype of total CD4$^+$ T cells from the same donors (B, D and E).

V) DISCUSSION

A Detection of CMV-specific CD8$^+$ T cells

1 Selection of streptamer multimer as tool for monitoring CMV-specific CD8$^+$ T-cell immune reconstitution in patients following allogeneic haematopoietic stem cell transplantation

CMV infection or reactivation represents a major complication in allo-HSCT recipients due to the delayed recovery of T- and B-cell functions that leads to impaired cytotoxic T-cell response (110). Development of a reliable method to evaluate CMV-specific immunity may facilitate the management of patients at risk for CMV infection/reactivation, allowing prompt initiation of antiviral treatment. In order to monitor disease-specific immune responses and to develop new immunotherapeutic strategies, it is essential to identify only those T cells that recognize a given MHC complex within the large pool of irrelevant T cells. In that sense, the use of multimer technologies (156) has become a useful tool in order to identify, quantify and monitor antigen-specific T-cells (155). Moreover, the use of multimer methodologies are nowadays being studied as new immunotherapeutic strategies to promote the reconstitution of antigen-specific immunity that could control viral infection following allo-HSCT (185).

The most common format in use to monitor antigen-specific T-cell recovery is tetramer multimer although other multimerization approaches such as PMs, octamers, dextramers and streptamers are available. Several studies use tetramer multimer in order to monitor CMV immune reconstitution in patients following allo-HSCT (77, 78). However, there is not, to our knowledge, any study that monitors CMV immune recovery in allo-HSCT recipients with other mulitmerization strategies that offer some advantages over classical tetramers (143). In this study, we have chosen and compared two of these advancements of multimer technologies, PM and ST. We chose PM due to the closeness to tetramer multimer configuration however, it results in higher avidity interaction with the TCR because of the same direction of the MHC-peptide complexes and brighter staining due to the five fluorochromes than tetramer does (159). By contrast, we selected ST as it can be dissociated from cytotoxic CD8$^+$ T-cell by adding the competitor molecule biotin and these ST-selected specific-CTLs can either be expanded or cloned and then adoptively be transferred to the patient (161, 163).

In order to select the best strategy for the incorporation into clinical monitoring practice, we first performed a detailed technical analysis of advantages and disadvantages of both technologies. In addition, the relative cost of using ST reagent was lower to the classical tetramer whereas the relative cost of PM multimer was similar to when considering its use for routine clinical monitoring.

Low standard deviation was observed with both multimer staining, showing a highly repeatability. Moreover, both methods showed low intra-individual variability, demonstrating that could be considered equally precise. Similar values of CMV-specific $CD8^+$ T cells were detected with both multimers and a significant correlation was detected between both techniques when frequencies more than 1% were detected but not a lower levels.

On the contrary, we have observed that PM technology detects higher amounts of multimer-positive $CD8^+$ T cells mainly at low dilutions when the sensitivity of these methodologies was studied. Our data differ from a previous study that reported similar results with both methodologies (186). We have observed that the PM technique is less accurate than the ST methodology, as the detected results with the PM multimer differed from the theoretical values. We confirmed these results by analysing multimer positive cells in non-HLA-A*02:01/CMV-seronegative, non-HLA-A*02:01/CMV-seropositive and HLA-A*02:01/CMV-seronegative volunteers, demonstrating that background signal levels detected were significantly higher with PM technology compared to ST methodology. This difference could be explained by the MFI observed for both multimers. PM staining provides lower MFI values than ST, and it is not easy to distinguish positive from negative populations when the proportion of specific cells is low (Figure 11). According to this, we can speculate that despite the binding affinity of MHC-TCR being the same for both multimers, the relative binding avidity may be different. This may, in turn, be higher for the ST multimer which makes a brighter staining.

Furthermore, some authors have studied non-specific binding on tetramers by comparing them to MHC mutated ones (187). It is widely known that in T-cell recognition there is not only an interaction between both the TCR and the peptide joint to the MHC complex but also peptide-MHC interactions with the cell surface CD8 that binds to invariable regions of the MHC class I molecules. As a result, a single MHC molecule can be bound simultaneously by both TCR and CD8 (188). The affinity of the TCR-MHC interaction

can be more than 100-fold stronger compared to the MHC-CD8 interaction (189). By modifying the MHC in the α3 domain, the CD8 binding to non-specific cells is abolished. These results suggest that the tetramer interacts with the co-receptor CD8 of T cells whose TCR had a low affinity for the peptide complex and could explain the background observed in our results with PM. The spatial configuration of the PM (similar to the tetramer) leads the α3 domain more available to the CD8 of non-specific cells allowing the tripartite TCR-MHC-CD8 interaction. This could be hindered in the ST due to the different structural configuration and composition (188).

The fact that ST is more reliable than PM especially at low frequencies of antigen-specific cells could be very useful in those patients that have little percentage of virus-specific CTLs that are not detected by other multimers. PM technology performed well when the monomeric TCR-MHC affinity was relatively high. However, it failed to detect T cells that are capable of recognizing a particular antigen, as T cells with weak, but functional TCRs might not be detected. Furthermore, due to unspecific bindings, positive staining could be obtained in the absence of virus-specific T cells. On the contrary, with the streptamer methodology, we could be able to monitor these specific T cells from the immediate post-transplant period, even when their frequencies are extremely low.

Other forms of multimerization such as octamers and dextramers also provide higher binding avidity than tetramers as they contain higher amounts of MHC molecules and fluorochromes. It is even possible to detect T cells with low TCR-MHC binding affinity with dextramers (190). However, octamers have been shown to activate the cytotoxic $CD8^+$ T cells for Fas dependent apoptosis (191). The use of tetramers for adoptive T-cell therapy has also been studied, however, their use as well as pentamers, octamers and dextramers offer a disadvantage over streptamer multimers as they cannot be dissociated from the antigen-specific $CD8^+$ T-cell. Functional status of multimer-selected antigen-specific T-cell might be hampered by the persistent binding of TCR-MHC interactions. By contrast, some side effects might be avoided with streptamer technology such as T-cell anergy, immune responses directly against the multimers, harm from clinical *in vivo* application and loss of the capacity of the transferred T cells to migrate *in vivo*, offering a new therapeutic approach at good manufacturing practice (192).

In conclusion, our results have shown that it is preferable to use ST technology that gives an accurate, precise and specific measurement to monitor the immune recovery of virus-

specific T cells in HSCT recipients. It also offers a potential use for adoptive T-cell therapy. However, it is necessary to point that these results do not represent either the entire CMV repertoire or HLA restrictions. We have selected the most common HLA allele within the Caucasian population (HLA-A*02:01) but the results may vary depending on the peptide-specificity or HLA restriction as avidity and affinity of them may depend on chemical structure. Some studies have pointed out this fact (77, 193). Therefore, future studies should be performed with other HLA restrictions and CMV epitopes to support our findings.

2 CMV-immune reconstitution after allogeneic haematopoietic stem cell transplantation

2.1 Features that contribute to CMV reactivation

CMV viremia is frequently seen within the first months following transplant and it is possible that allo-reactive immune responses contribute in some way to the process of reactivation.

In the present study, we have evaluated T-cell immune reconstitution against CMV infection in 25 HLA-A*02:01 recipients during one year post allo-HSCT. T-cell recovery as well as CMV-specific $CD8^+$ T-cell response have been analysed in order to predict the kinetics of CMV immune reconstitution. In our study we have monitored patients by using two different methodologies: ST multimer and *in vitro* IFNγ production to assess CMV-specific $CD8^+$ T-cell recovery.

In our cohort of patients, the cumulative incidence of CMV reactivation during the first year after allo-HSCT was not influenced by HLA compatibility as Jaskula *et al.* and Mead *et al.* observed in their studies that a lack of optimal donor/recipient HLA matching was associated with higher CMV reactivation or infection (68, 79). Conditioning treatment did not have any impact on CMV reactivation either as it has been shown by other researchers where CMV reactivation was less common in patients receiving RIC conditioning compared to MA treatment (84). On the contrary, CMV reactivation was significantly influenced by both HSCT donor type being more frequent in patients receiving transplant from unrelated donors, and T-cell depletion in conditioning treatment. Both data are related as *in vivo* T-cell depletion is administered in patients receiving unrelated donor transplant to reduce GvHD and thus, there is a delay on immune reconstitution. Our findings agree with previous studies by Lilleri and collaborators, where CMV infection/reactivation was more frequent in people receiving a T-cell depleted graft (71).

Previous studies have shown the impact that donor CMV serostatus has on CMV reactivation after allo-HSCT. In that sense, CMV seropositive patients whose donors were CMV seronegative experienced a higher risk of CMV reactivation, compared to those whose donors were seropositive (68, 71). In our cohort of patients all but one were CMV seropositive, however 32% received a transplant from a seronegative donor. In our

cohort of patients, donor CMV-serology did not have any influence on CMV reactivation within our patients. This fact might be due to the low number of patients that could be monitored.

2.2 T-cell immune recovery may influence on CMV reactivation following transplant

The development of an effective T-cell immunity is important for the control of CMV infection/disease in patients following allo-HSCT. Previous studies have found a strong association between the lack of CMV-specific $CD8^+$ T cells and the development of CMV disease after transplantation. Therefore the identification of the protective cell number against CMV infection and reactivation is of special interest.

In our cohort of patients, normal median values of both $CD4^+$ and $CD8^+$ T cells (>100 cells/μL and >50 cells/μL, respectively) were reached after two months of transplantation in patients that did not experience CMV-DNAemia, whereas recipients with CMV reactivation and short antiviral treatment reached those levels between two and three months post-transplantation. On the contrary, recipients that required prolonged antiviral treatment achieved those levels between 200 days and 300 days after allo-HSCT and they have experienced CMV reactivations after 100 days following transplant. Recurrent $CD4^+$ T-cell counts lower than 100 cells/μL during the first three months has been strongly associated with CMV reactivation, underscoring the adverse effect of impaired $CD4^+$ T-cell reconstitution on infectious morbidity and late CMV disease (194, 195). Our results are supported by other authors, who estimated that cut-off values for $CD4^+$ less than 100 cells/μL and $CD8^+$ less than 50 cells/μL at three months after transplantation were associated with poor CMV specific immunity (196). These results agree with those obtained by Moins-Teisserenc and colleagues, in which they suggested that T-cell counts higher than 100 cells/μL for both $CD4^+$ and $CD8^+$ may prevent the risk of CMV reactivation (197).

We have observed a good correlation between CMV-specific $CD8^+$ T cells absolute counts regardless of CMV reactivation status and total $CD8^+$ and $CD4^+$ counts in our cohort of patients. These observations differ from those found by Gratama *et al.*, where CMV-CTL levels showed a moderate correlation with total $CD8^+$ T-cell counts but a negligible correlation with absolute $CD4^+$ counts (157). These results suggested that

$CD4^+$ T-cell function might be essential for CMV-specific $CD8^+$ T-cell immune reconstitution.

2.3 CMV-specific $CD8^+$ T-cell expansion after CMV reactivation prevents multiple CMV reactivations and affects antiviral treatment

Similar to total $CD8^+$ and $CD4^+$ levels to prevent CMV reactivation, a protective number of CMV-specific $CD8^+$ T cells has been suggested by several authors to avoid CMV reactivation and disease. In that sense, Borchers and colleagues (77) proposed that levels of 1 CMV-specific $CD8^+$ T cell/μL could protect against CMV reactivation when monitoring patients following allo-HSCT by tetramer multimer. However, it is interesting to mention that protective cell numbers may vary considerably for individual combinations of HLA molecules and CMV epitopes.

In our study, no differences were found between levels of CMV-specific $CD8^+$ T cells detected by the multimer technique when comparing patients without and with CMV reactivation at early time points after allo-HSCT (day +30). Besides, the median number of CMV-specific $CD8^+$ T cells detected with the ST multimer was 0.780 cells/μL (0.050-1.080) during the year follow up in patients that did not reactivate. Those levels are similar to the ones found to be protective in immunocompetent subjects (more than 0.4 CMV-specific T cells/μL of blood) and therefore patients were able to control CMV infection without the need of antiviral treatment (71). However, we cannot use either 0.4 or 1 CMV-specific $CD8^+$ T cells/μL as a cut-off value for all the cohort of patients, as we have observed CMV reactivations in several recipients despite the presence of 1 CMV-specific $CD8^+$ T cell/μL. We have evaluated patients that experienced CMV viremia according to duration of antiviral treatment. Patients with short antiviral treatment developed CMV-CTL proliferation as measured by an increase on multimer positive cells after first CMV reactivation. This expansion of CMV-specific $CD8^+$ T cells does correlate to protection against recurrent CMV reactivations after 100 days following transplant, while the absence of CMV-specific immune reconstitution leads to recurrent reactivation of CMV beyond day +100 post-transplant and prolonged antiviral therapy. The results published by Borchers and collaborators and Moins-Teisserenc *et al.* support our findings, as they found that non-proliferating CMV-CTL post CMV reactivation correlated with recurrence (78, 197).

In addition to currently controversial CMV-CTL quantity that provides protection against viral reactivation, the influence of CMV reactivations on immune reconstitution is also widely debated. While Chen *et al.* argue that CMV reactivation boots the reconstitution of CMV-specific CTLs (198), others find no influence of CMV reactivation on CMV-CTL reconstitution (199). In our study, patients reactivating CMV had higher median CMV-CTL numbers than patients without CMV reactivations, implying a significant influence of CMV reactivation on levels of CMV-CTLs detected. Besides, we have observed a group of recipients within patients that required short antiviral treatment that only had one episode of CMV-DNAemia, whereas others had two or three CMV reactivations. Levels of CMV-specific $CD8^+$ T cells detected after the first CMV reactivation were significantly higher in those patients compared to recipients with more than one reactivation. Interestingly, no differences were found in CMV-CTL levels after CMV reactivation in patients with only one reactivation, or the second reactivation in patients that reactivated twice or after the third reactivation in those with 3 episodes of CMV-DNAemia. The lowest number of CMV-specific $CD8^+$ T cells reached after CMV reactivation in our cohort of individuals was 1.348 CMV-CTLs/μL and therefore, it could be protective threshold against recurrent CMV reactivation. Patients without CMV viremia also expanded CMV-CTLs, but to a lesser extent than those with CMV reactivation, as it was observed by the increase of those cells between days +30 and +60. Borchers *et al.'s* study, carried out in almost 200 patients, supported our findings (77). However, it is necessary to point out that CMV-CTL numbers may vary for individual combinations of HLA molecules and CMV epitopes as reported by Borchers and colleagues (77).

In summary, CMV ST-based immune monitoring, in conjunction with virology monitoring, can be an important new tool that permits clinicians to assess the risk of CMV-related complications and to guide pre-emptive therapeutic choices. Indeed, sequential monitoring of the post-transplant CMV-CTL immune reconstitution allows a more accurate interpretation of an individual patient's response to CMV. In addition, we have demonstrated that expansion of CMV-specific $CD8^+$ T cells after CMV reactivation adds to the protection against recurrent CMV reactivations whereas non-proliferating CMV-CTL post CMV reactivation correlated with recurrence. However, as there is a group of patients who did not develop CMV-DNAemia and CMV-CTL levels hardly expanded there must be other factors involved in this protection.

2.4 Functional recovery of CMV-specific CD8$^+$ T cells after transplantation prevents CMV reactivation

We were further interested in studying the group of patients that did not reactivate CMV despite not reaching the cut-off value for CMV-specific CD8$^+$ T cells. In spite of finding no differences in the number of CMV-specific CD8$^+$ T cells detected by multimer technology between patients with and without CMV reactivation at early time points (day +30) after allo-HSCT, significant differences were found in the functionality of those cells. CMV-CTLs from patients that never experienced CMV viremia were functional one month after transplantation, whereas functional recovery did not occur in recipients that developed CMV reactivation. Therefore, having functional CMV-specific CD8$^+$ T cells at early time points following transplant may protect against CMV reactivation. The median number of *in vitro* IFNγ$^+$CD8$^+$ T cells of those recipients during the year follow up was 0.630 cells/μL (0.094-55.159), which is closer to the protecting levels obtained in healthy individuals (>0.4 cells/μL) (71).

Most CMV reactivations occurred between the first and the second month after allo-HSCT in our cohort of patients and thus, we analysed the functionality of CMV-specific CD8$^+$ T cells two months post-transplantation. As observed with multimer technology, there was an increase of *in vitro* IFNγ production in patients that experienced CMV-DNAemia, compared to recipients without CMV reactivation. This fact indicates that CMV reactivation induces expansion of functional CMV-CTL.

In our study, patients receiving short antiviral treatment had median levels of functional CMV-CTLs significantly lower in the presence of CMV-DNAemia than at the time of the first negative PCR result. This supports the fact that CMV reactivation induces T-cell expansion. However, despite increasing the numbers of functional CMV-CTLs after reactivation, there are patients with more than one CMV reactivation. Therefore, not only the expansion, but also reaching threshold levels may be a prerequisite to resolve CMV reactivation. Taking into consideration the lowest value for IFNγ$^+$CD8$^+$ T cells found after CMV viremia clearance in this group, we can suggest 1.290 cells/μL as a threshold value for predicting CMV-DNAemia clearance (negative PCR result). These cut-off level is remarkably closed to those previously determined by using functional assays for IFNγ and IL-2 secretion or ELISpot assays in a higher cohort of individuals by Tormo *et al.* (>1.3 CMV-CTLs/μL) (200) and by other groups (1-3 CMV- CTLs/μL) (86, 201, 202).

This implies that detection of 1 to 3 CMV-CTLs/μL of blood may indicate the threshold of a functional immunity against CMV.

The inability to control CMV reactivation is related to functional impairment of antigen-specific $CD8^+$ T cells. This fact has been demonstrated by Gratama *et al.* and Morita-Hoshi. *et al*. (203, 204), and it may explain the behaviour of those patients requiring prolonged antiviral treatment in our cohort of recipients. There is no IFNγ production by $CD8^+$ T cells upon *in vitro* stimulation with CMV-$pp65_{495-503}$ antigen and several CMV reactivations are developed that are controlled by antiviral therapy. These patients did not show either CMV-CTLs when using multimer technology or *in vitro* IFNγ production upon stimulation with CMV-$pp65_{495-503}$ antigen during the first months after allo-HSCT. After 200 days approximately following transplant, functional activity is recovered and these patients stop CMV reactivations.

The use of a methodology that enables the detection of cytokines produced by virus-specific cells upon *in vitro* stimulation would be very useful in monitoring the functionality that those specific cells may have. We have measured the IFNγ production by CMV-specific cells by using a γ-capture-based kit according to manufacturer's instructions. It is interesting to mention that the differences that we detected on *in vitro* IFNγ production with this technology compared to ICS technique could be because of prelabelling the T cells with the pentamer multimer that may partially inhibit their ability to respond to the target peptide as it has been observed with other multimers (163, 192). Unfortunately, it has not been possible to optimize and use this technique to monitor functionality of multimer$^+$$CD8^+$ T cells due to the low number of patients that could be analysed. Further studies would be necessary in order to evaluate its use for the monitoring of functional antigen-specific T-cell recovery in patients following transplant.

On the basis of our results, we have observed three different kinetic patterns of CMV-specific $CD8^+$ T-cell reconstitution:

(i) An early recovery of functional CMV-specific CTLs that might protect from CMV reactivation.

(ii) An expansion of functional CMV-specific $CD8^+$ T cells is observed after CMV reactivation. Those patients with increasing CMV-specific CTLs detectable at the time of PCR reactivation only need a short course of antiviral therapy.

(iii) An inconsistent or a lack of expansion of functional CMV-specific $CD8^+$ T cells with recurrent CMV-DNAemia and in need of prolonged antiviral therapy.

In summary, our data suggest that routine immunological monitoring following transplant allows a more accurate interpretation of an individual patient's response to CMV. The protective hallmarks might depend on reactivation status. Analysis of the CMV-CTL expansion may facilitate implementation of patient-specific antiviral therapy.

In addition, our results have shown that not only the number of CMV-specific $CD8^+$ T cells may be related to CMV reactivation but also their ability to respond to CMV through either CMV-specific T-cell expansion or IFNγ production which can confer protection against CMV reactivation. These observations agree with a recent study developed by Suessmuth Y and colleagues (205) in which it is shown that CMV reactivation drives post-transplant CMV-specific T-cell reconstitution and results in defects in the underlying TCRβ repertoire.

2.5 Future perspectives

The use of class I multimer technology allows direct measurement of the frequency of virus-specific T cells. There are kits available to detect CMV-specific T cells in whole blood by using dextramer technology that can be implemented in the monitoring of CMV immune recovery after allo-HSCT (190).

Because CMV reactivation arises from impaired CMV-specific T-cell immunity, various adoptive transfer protocols have been developed in order to restore cellular immunity against CMV (120, 206). The main purpose of antiviral adoptive immunotherapy is to

provide a competent immunological response able to control the virus. Many groups have focused their interest on developing strategies for adoptive transfer of CMV-specific T cells. The isolation of CMV-specific CTLs by using ST technology has shown that these cells retain their effector function which is essential for a successful adoptive immunotherapy (192). Some clinical studies have been developed using this technology as ST is available at a good manufacturing practice level (185) (https://www.clinicaltrials.gov/ct2/NCT01077908/ or NCT01220895 or NCT01220895).

According to our data, a lack of consistent expansion of CMV-specific T cells is associated with persistent CMV DNAemia, even in the absence of proven resistance to antivirals. In this regard, it would be reasonable to consider adoptive transfer of CMV-specific functional T cells as a therapeutic option in episodes of active CMV infections that do not respond to antiviral therapy. (185, 207).

Therefore, the analysis of the CMV-CTL expansion rate may facilitate implementation of patient-specific antiviral strategies, including adoptive transfer of CMV-CTLs to recipients unable to respond to CMV reactivation.

B Phenotypic characterisation of CMV-specific CD4$^+$ T cells

1 Identification of CMV-specific CD4$^+$ T cells by MHC class II tetramers

For more than a decade, there is a debate on whether both CMV-specific CD4$^+$ and CD8$^+$ are required to confer protection against CMV reactivation, or whether only one of these two T-cell populations is sufficient to protect from CMV relapse. Some authors have indicated that CMV-specific CD4$^+$ T cells may be sufficient to predict a reliable control of CMV infection (55, 203). By contrast, recent studies have shown that both T-cell subsets are required for a long-lasting protection against CMV reactivation (71, 200, 208). Moreover, effective control of CMV infection was attained in patients when CMV-specific cells, were infused, of which 77% were CD4$^+$ T cells (186). Gamadia *et al.* reported that during primary CMV infection, virus-specific CD4$^+$ T cells precede the appearance of virus-specific CD8$^+$ T cells in renal transplant recipients (43). Both the absence of antigen-specific CTLs, and particularly the absence of specific CD4$^+$ T helper cells resulted in higher CMV loads (209). Thus, CD4$^+$ T-helper cells are likely to participate in CMV control, as it has been observed, in out cohort of individuals, in the correlation between CMV-specific CD8$^+$ T cells and CD4$^+$ T cells.

The presence of CMV-specific CD4$^+$ T-cell clones has been reported based on cytokines produced upon *in vitro* stimulation with CMV peptides, virus lysate and CMV protein (210). CD4$^+$ T cells usually recognize an antigen that has been taken up by the antigen presenting cell rather than presented directly from cytosol. Kern *et al.* reported that there is a high prevalence of CD4$^+$ T-cell responses to pp65, and peptides derived from tegument proteins are likely to be presented in a MHC class II context after endocytosis or phagocytosis of viral material (37). Recent evidence has indicated that CMV gB is sorted to endosomes and can be presented efficiently by class II molecules which is especially relevant as it can directly be presented by CMV-infected cells without the need to be taken up by APCs(211).

The study of antigen-specific CD4$^+$ T cells by using class II multimer technology has been limited by the insensitivity of detection in *ex vivo* samples and paucity of defined CD4$^+$ T-cell epitopes with known HLA restriction. Another limiting factor is simply technical issues in making them, as MHC class II are structurally different when compared with class I. In the last years, more epitopes have been defined, so the utility of

HLA class II tetramers is expanding (170). CMV-MHC class II tetramers have been previously studied without successful results, suggesting that due to the low frequencies it was necessary an enrichment step by using magnetic beads to allow accurate definition of a responder population, as it occurs with other virus such as EBV (171, 212).

In our study, we have described for the first time, three MHC class II tetramers to identify CMV-specific $CD4^+$ T cells directly *ex vivo* without the need of an enrichment step, allowing a reliable detection of CMV-specific $CD4^+$ T cell. Besides, CMV-specific $CD4^+$ T-cell response compared to other antigen-specific $CD4^+$ T-cell responses, for example those to EBV) can be extremely large (although not in every donor) and it can be at least as marked as that seen within the $CD8^+$ T-cell populations. Besides, the magnitudes of DYS-, AGI- and LLQ-specific $CD4^+$ T cell responses averages from 0.07 to 24% of the total $CD4^+$ T-cell repertoire and are much higher than those obtained by Harcourt *et al.* even after enrichment (171). However, we have only targeted a very limited HLA repertoire and there may be other dominant epitopes. The presentation of peptides through class II molecules could be of particular importance in control of CMV and other herpesviruses as these pathogens have developed immune evasion strategies to avoid recognition by $CD8^+$ T cells and NK cells (213).

2 Cytotoxic function and effector memory phenotype of CMV-specific $CD4^+$ T cells

Over the last two decades, the ability of $CD4^+$ T cells to display cytotoxic potential has been reported by several studies in humans. Nevertheless, the observation of such cytotoxic activity in $CD4^+$ T cells has usually been restricted to cell lines and $CD4^+$ T-cell clones generated by long term *in vitro* culture and it has been considered an artefact by some authors (214). However, Appay *et al.* were the first to report the presence of $CD4^+$ T cells with cytotoxic capacity without the need for *in vitro* stimulation in humans (144). In our study, we have also observed cytotoxic $CD4^+$ T cells *ex vivo* as there is a population within the $CD4^+$ T-cell subset that express the cytotoxic molecules perforin and granzyme B in healthy individuals. Appay *et al.* suggested that the presence of these cells was related to chronic viral infections. Interestingly, the direct staining with tetramer multimers has shown that the vast majority of CMV-specific $CD4^+$ T cells showed this cytotoxic potential by expressing both perforin and granzyme B molecules. It is interesting to mention that this high cytotoxic potential varies depending on their peptide

specificity. DYS-specific $CD4^+$ T cells present higher levels of both granzyme B and perforin than AGI- and LLQ-specific $CD4^+$ T cells (39, 62).

The phenotypic analysis of the tetramer-positive $CD4^+$ T cells revealed that CMV-specific $CD4^+$ T cells showed predominantly an effector memory phenotype characterised by a complete loss of CCR7. Most cells expressed the memory marker CD45RO (this isoform is associated with antigen-experienced cells), although a minority had a revertant memory phenotype with high-level expression of CD45RA (T_{EMRA}), which has only been observed on the DYS-specific $CD4^+$ T-cell population (215, 216). The opposite is seen on CMV-specific $CD8^+$ T cells that have been widely described to be mainly of the late differentiated effector memory phenotype (EMRA) (217-219).

It has been observed that differentiating $CD4^+$ T cells first lose expression of CD27 and only in a later phase they lose CD28, thus all $CD28^-$ are $CD27^-$. It is the opposite for $CD8^+$ T cells, where $CD8^+CD27^-$ cells always have lost CD28 expression (39). The presence of $CD4^+CD28^-$ T cells is not commonly seen and CMV infection is the major factor causing this differentiation step of $CD4^+$ T cells (39). Besides, these cytotoxic $CD4^+CD28^-$ cells display increased expression levels of CD57, which is a marker of replicative incompetence and it has been associated with a highly differentiated cytotoxic phenotype. Our results reported that CMV-specific $CD4^+$ T cells present differences in their differentiation profile depending on the CMV protein-recognized (gB or pp65). gB-(DYS) specific to the same extent as pp65-(AGI/LLQ) specific $CD4^+$ T cells have lost CD27 surface expression marker; however, CMV-specific $CD4^+$ T cells that recognize peptides from the CMV-pp65 protein, AGI and LLQ, presented higher levels of CD28 co-stimulatory molecule and also had lower levels of CD57 compared to DYS-specific $CD4^+$ T cells that showed a highly differentiated phenotype. We have also observed that this differentiation status of DYS-specific $CD4^+$ T cells was related to age (30).

This progressive differentiation with the loss of CD27 and CD28 molecules has been associated with the expression of intracellular cytotoxic granules and perforin, as observed with $CD8^+$ T cells. Our analyses reported that both AGI- and LLQ-specific $CD4^+$ T cells presented lower levels of perforin compared to DYS-specific $CD4^+$ T cells which agree with higher expression of CD28 on pp65-epitope-specific $CD4^+$ T cells. Therefore, highly differentiated CMV-specific $CD4^+$ T cells become cytotoxic by the expression of perforin at the CD28 negative stage. We have observed that DYS-specific

cells are highly cytotoxic compared to AGI- and LLQ-specific cells probably due to the differences on the antigen process.

Therefore, these results showed that CMV-specific $CD4^+$ T cells are highly cytotoxic *ex vivo* and it varies depending on their peptide specificity. Further, these cells are mainly effector memory cells, with a late differentiation stage characterise by the loss of CD28 co-stimulatory molecule. A possible explanation of the different phenotype between DYS- and AGI- or LLQ-specific $CD4^+$ T cells could explain by the differences in priming of the response. Both proteins, gB and pp65, are quite abundant in the virion and also expressed late in the virus life cycle. gB is directed into the MHC class II pathway and directly presented, rather than indirectly by antigen presenting cells like pp65. Therefore, gB-specific T cells may be more driven towards a more "differentiated" phenotype.

Similar to NK cells and cytotoxic $CD8^+$ T cells, we have observed that CMV-specific $CD4^+$ T cells express the killer lectin receptor NKG2D. These observations were also reported in a study by Sáez-Borderías *et al*. where an increase on NKG2D expression by $CD4^+$ T cells after 10 days of *in vitro* stimulation with CMV virus was observed (180). We detected the same levels of NKG2D expression on CMV-specific $CD4^+$ T cells by direct staining with multimers than they obtained after 10 days of stimulation, approximately, 20% of the CMV-specific $CD4^+$ T cells expressed this receptor. Their study also suggested that the expansion of $CD4^+$ T cells expressing NKG2D after stimulation corresponded to virus-specific memory cells that have acquired NKG2D while losing CD28. It is possible that the lack of co-stimulatory molecules such as CD27 and CD28 can be overcome by activation through binding of activating NK cell receptors such as NKG2D with its specific ligand that is expressed on infected cells. Unfortunately, we have not included all three markers in the same staining panel to analyse the co-expression of NKG2D and CD28, but NKG2D expression tends to increase while CD28 decreases on cells detected in the same individual.

Of note, T-cell exhaustion which is characterised by the up-regulation of inhibitory receptors, such as PD-1, and the progressive loss of cytokine production, proliferative capacity, and cytolytic function (220) has not been found applicable for CMV-specific $CD8^+$ T cells (184, 221). It has, although been associated with other chronic viral infections mainly those where there were high levels of antigen like Human Immunodeficiency virus and Hepatitis C virus. Interestingly, the staining *ex vivo* with class II tetramer methodology has shown that a great proportion of CMV-specific $CD4^+$

T cells expressed PD-1 molecule in comparison with total $CD4^+$ T cells. Our findings are supported by Sester *et al.*'s study, in which they observed expression of PD-1 on CMV-specific $CD4^+$ T cells after 5 days of *in vitro* stimulation with CMV antigen (222). This functional exhaustion of CMV-specific $CD4^+$ T cells may determine impaired CMV control in patients following transplantation. Unfortunately, we have not performed functional assays with these cells, but previous studies have shown that PD-1 positive cells are less functional with lower IFNγ and IL-2 levels compared to PD-1 negative cells. This functional anergy of CMV-specific $CD4^+$ T cells can be avoided by blocking PD-1 signalling with its ligands PD-L1/L2 which has been shown to increase up to 10-fold on CMV-specific $CD4^+$ T cells therefore, providing a potential target for enhancing the function of exhausted T cells in chronic CMV infection (222). Interestingly, PD1 expression was reduced on DYS-specific $CD4^+$ T cells compared to AGI- and LLQ-specific $CD4^+$ T cells. Further analyses need to be perform in order to understand why it occurred as it is the opposite to what it was expected since DYS-specific $CD4^+$ T cells presented a highly differentiated phenotype and were thought to be more exhausted than AGI- and LLQ-specific $CD4^+$ T cells.

Another interesting observation that our study has revealed is that CMV-specific $CD4^+$ T cells staining directly *ex vivo* do not appear to belong to the $CD4^+$ T regulatory subset as these cells did not express neither CD25 surface marker nor FoxP3 transcriptor factor. Appay *et al.* came to the same conclusion when analysing cytotoxic $CD4^+$ T cells (144). However, two previous studies showed the presence of inducible regulatory T cells within CMV-specific $CD4^+$ T cells, although it was observed upon *in vitro* stimulation with CMV peptides or CMV lysate and in our study we performed a direct staining (223, 224).

As it has previously been mentioned, CMV establishes latency in various cell types, including myeloid lineage cells but also endothelial cells. Activated endothelial cells upon inflammatory processes produce cytokines such as fractalkine that can recruit immune cells that are able to induce endothelial damage (225, 226). The receptor for fractalkine is CX3CR1, which has been shown to be expressed on macrophages, dendritic cells and fibroblasts (183). Recent studies have shown that CX3CR1 is only expressed on CMV-specific effector $CD8^+$ T cells when compared to EBV and influenza specific T cells (184). Interestingly, our results have shown that the majority of CMV-specific $CD4^+$ T cells also expressed the fractalkine receptor. These cells also have high levels of granzyme B and perforin, which can cause tissue damage to the endothelial cells. The

potential of the host antigen-specific T-cell population to respond aggressively to CMV with a great cytokine response may initiate a chemokine cascade and direct an inflammatory infiltrate that has a self-destructive effect on the endothelium. Endothelial-cell damage may be the end effect of a repeated cycle in which the cell and tissue damage results from innate immune defence mechanisms (monocyte-macrophage cells) activated by a strong T-cell response to a chronic pathogen that is present as either a persistent infection or a latent infection undergoing periodic reactivation episodes. This may be a problem associated with many inflammatory disorders if they involve a chronic pathogen such as CMV, with which the antigen-specific T-cell response can result in chemokine induction and lead to chronic inflammation. Breaking the cycle of antigen presentation to the T cells by restricting virus replication may help to limit or even reverse the development of this aggressive T-cell response to CMV (226).

In conclusion, we have demonstrated that MHC class II tetramers allow reliable detection of CMV-specific $CD4^+$ T cells. These cytotoxic CMV-specific $CD4^+$ T cells that appear as a consequence of CMV infection may have a considerable importance in the clearance of viral infection and bear many phenotype similarities to their cytotoxic CD8 counterparts. Antigen-presenting cell populations have constitutive expression of HLA class II proteins, whereas other cell types, such as endothelium, are induced to express HLA class II molecules by inflammatory mediators. These cell types are tropic for CMV infection, and thus become targets for CD4 mediated immune control. The role of $CD4^+$ T cells and their recognition of MHC class II may be critical for activating the immune system and sustaining the balance between virus and host immunity during latency.

We have been able to describe the phenotype of CMV-specific $CD4^+$ T cells by direct staining *ex vivo* by using class II tetramers in healthy individuals and it is now possible to implement this technique for the monitoring of CMV immune recovery in allo-HSCT recipients, as it has been shown that effector memory $CD4^+$ T cells are necessary for recovery of infection. By using class II tetramers, it would possible to study CMV-specific $CD4^+$ T-cell responses in transplant patients and it would help in the knwoledge of CMV reactivation. It would be possible to sort CMV-specific $CD4^+$ T cells from those patients in order to perform molecular assays to the better understanding of CMV infection.

VI) CONCLUSIONS

1. Streptamer multimer gives an accurate, precise and specific measurement in order to monitor immune recovery of CMV-specific $CD8^+$ T cells compared to pentamer multimer.

2. $IFN\gamma^+$ CMV-CTL levels at early time points following transplant protects patients against CMV reactivation.

3. CMV-CTL expansion levels after CMV reactivation protects against future reactivations in allogeneic haematopoietic stem cell transplant recipients.

4. CMV-CTLs monitoring during episodes of CMV DNAemia yield useful information for the therapeutic management of active CMV infection in allogeneic haematopoietic stem cell transplant recipients.

5. MHC class II tetramers allow reliable detection of CMV-specific $CD4^+$ T cells.

6. CMV-specific $CD4^+$ T cells are highly cytotoxic *ex vivo* and these cytotoxic potential varies on the peptide-specificity.

7. Cytotoxic CMV-specific $CD4^+$ T cells present mainly an effector memory phenotype and present a differentiated phenotype which varies depending on peptide-specificity.

VII) REFERENCES

1. Alberts B, J. A., Lewis J, et al. 2002. Innate Immunity. In *Molecular Biology of the Cell*.

2. Ozaki, K., and W. J. Leonard. 2002. Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem* 277: 29355-29358.

3. Alberts B, J. A., Lewis J, et al. 2002. *The Adaptive Immune System.*

4. Choo, S. Y. 2007. The HLA system: genetics, immunology, clinical testing, and clinical implications. *Yonsei medical journal* 48: 11-23.

5. Alberts B, J. A., Lewis J, et al. . 2002. *T Cells and MHC Proteins.*

6. Santana, M. A., and F. Esquivel-Guadarrama. 2006. Cell biology of T cell activation and differentiation. *International review of cytology* 250: 217-274.

7. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7: 145-173.

8. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nature immunology* 8: 345-350.

9. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22: 531-562.

10. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science* 272: 54-60.

11. Heath, W. R., and F. R. Carbone. 2001. Cross-presentation in viral immunity and self-tolerance. *Nature reviews. Immunology* 1: 126-134.

12. Ljungman, P., M. Bregni, M. Brune, J. Cornelissen, T. de Witte, G. Dini, H. Einsele, H. B. Gaspar, A. Gratwohl, J. Passweg, C. Peters, V. Rocha, R. Saccardi, H. Schouten, A. Sureda, A. Tichelli, A. Velardi, and D. Niederwieser. 2010. Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe 2009. *Bone Marrow Transplant* 45: 219-234.

13. Gratwohl, A., H. Baldomero, M. Aljurf, M. C. Pasquini, L. F. Bouzas, A. Yoshimi, J. Szer, J. Lipton, A. Schwendener, M. Gratwohl, K. Frauendorfer, D. Niederwieser, M. Horowitz, and Y. Kodera. 2010. Hematopoietic stem cell transplantation: a global perspective. *Jama* 303: 1617-1624.

14. Copelan, E. A. 2006. Hematopoietic stem-cell transplantation. *N Engl J Med* 354: 1813-1826.

15. Shaw, B. E., D. L. Confer, W. Y. Hwang, D. H. Pamphilon, and M. A. Pulsipher. 2011. Concerns about the use of biosimilar granulocyte colony-stimulating factors for the mobilization of stem cells in normal donors: position of the World Marrow Donor Association. *Haematologica* 96: 942-947.

16. Passweg, J. R., H. Baldomero, M. Bregni, S. Cesaro, P. Dreger, R. F. Duarte, J. H. Falkenburg, N. Kroger, D. Farge-Bancel, H. B. Gaspar, J. Marsh, M. Mohty, C. Peters, A. Sureda, A. Velardi, C. Ruiz de Elvira, and A. Madrigal. 2013. Hematopoietic SCT in Europe: data and trends in 2011. *Bone Marrow Transplant* 48: 1161-1167.

17. Bensinger, W. I. 2012. Allogeneic transplantation: peripheral blood vs. bone marrow. *Current opinion in oncology* 24: 191-196.

18. Ballen, K. K., E. Gluckman, and H. E. Broxmeyer. 2013. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood* 122: 491-498.

19. Aschan, J. 2006. Allogeneic haematopoietic stem cell transplantation: current status and future outlook. *British medical bulletin* 77-78: 23-36.

20. Socie, G., R. A. Clift, D. Blaise, A. Devergie, O. Ringden, P. J. Martin, M. Remberger, H. J. Deeg, T. Ruutu, M. Michallet, K. M. Sullivan, and S. Chevret. 2001. Busulfan plus cyclophosphamide compared with total-body irradiation plus cyclophosphamide before marrow transplantation for myeloid leukemia: long-term follow-up of 4 randomized studies. *Blood* 98: 3569-3574.

21. Jimenez, M., G. Ercilla, and C. Martinez. 2007. Immune reconstitution after allogeneic stem cell transplantation with reduced-intensity conditioning regimens. *Leukemia* 21: 1628-1637.

22. Blaise, D., and L. Castagna. 2012. Do different conditioning regimens really make a difference? *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* 2012: 237-245.

23. Landolfo, S., M. Gariglio, G. Gribaudo, and D. Lembo. 2003. The human cytomegalovirus. *Pharmacology & therapeutics* 98: 269-297.

24. van de Berg, P. J., A. van Stijn, I. J. Ten Berge, and R. A. van Lier. 2008. A fingerprint left by cytomegalovirus infection in the human T cell compartment. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 41: 213-217.

25. Khan, N. 2007. The immunological burden of human cytomegalovirus infection. *Archivum immunologiae et therapiae experimentalis* 55: 299-308.

26. Slezak, S. L., M. Bettinotti, S. Selleri, S. Adams, F. M. Marincola, and D. F. Stroncek. 2007. CMV pp65 and IE-1 T cell epitopes recognized by healthy subjects. *Journal of translational medicine* 5: 17.

27. Elkington, R., N. H. Shoukry, S. Walker, T. Crough, C. Fazou, A. Kaur, C. M. Walker, and R. Khanna. 2004. Cross-reactive recognition of human and primate cytomegalovirus sequences by human CD4 cytotoxic T lymphocytes specific for glycoprotein B and H. *European journal of immunology* 34: 3216-3226.

28. Braendstrup, P., B. K. Mortensen, S. Justesen, T. Osterby, M. Rasmussen, A. M. Hansen, C. B. Christiansen, M. B. Hansen, M. Nielsen, L. Vindelov, S. Buus, and A. Stryhn. 2014. Identification and HLA-Tetramer-Validation of Human CD4+ and CD8+ T Cell Responses against HCMV Proteins IE1 and IE2. *PloS one* 9: e94892.

29. Khan, N., N. Shariff, M. Cobbold, R. Bruton, J. A. Ainsworth, A. J. Sinclair, L. Nayak, and P. A. Moss. 2002. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *Journal of immunology* 169: 1984-1992.

30. Pourgheysari, B., N. Khan, D. Best, R. Bruton, L. Nayak, and P. A. Moss. 2007. The cytomegalovirus-specific CD4+ T-cell response expands with age and markedly alters the CD4+ T-cell repertoire. *J Virol* 81: 7759-7765.

31. La Rosa, C., and D. J. Diamond. 2012. The immune response to human CMV. *Future virology* 7: 279-293.

32. Wills, M. R., A. J. Carmichael, K. Mynard, X. Jin, M. P. Weekes, B. Plachter, and J. G. Sissons. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J Virol* 70: 7569-7579.

33. Gerna, G., D. Lilleri, D. Caldera, M. Furione, L. Zenone Bragotti, and E. P. Alessandrino. 2008. Validation of a DNAemia cutoff for preemptive therapy of cytomegalovirus infection in adult hematopoietic stem cell transplant recipients. *Bone Marrow Transplant* 41: 873-879.

34. Sellar, R. S., and K. S. Peggs. 2012. Therapeutic strategies for the prevention and treatment of cytomegalovirus infection. *Expert Opin Biol Ther* 12: 1161-1172.

35. Busca, A. 2009. Cytomegalovirus (CMV) infection after hematopoietic stem cell transplantation: significant progress, but many unresolved problems. *Expert Opin Biol Ther* 9: 383-385.

36. Rossini, G., C. Cerboni, A. Santoni, M. P. Landini, S. Landolfo, D. Gatti, G. Gribaudo, and S. Varani. 2012. Interplay between human cytomegalovirus and intrinsic/innate host responses: a complex bidirectional relationship. *Mediators of inflammation* 2012: 607276.

37. Kern, F., T. Bunde, N. Faulhaber, F. Kiecker, E. Khatamzas, I. M. Rudawski, A. Pruss, J. W. Gratama, R. Volkmer-Engert, R. Ewert, P. Reinke, H. D. Volk, and L. J. Picker. 2002. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *The Journal of infectious diseases* 185: 1709-1716.

38. Waldrop, S. L., C. J. Pitcher, D. M. Peterson, V. C. Maino, and L. J. Picker. 1997. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *The Journal of clinical investigation* 99: 1739-1750.

39. van Leeuwen, E. M., E. B. Remmerswaal, M. T. Vossen, A. T. Rowshani, P. M. Wertheim-van Dillen, R. A. van Lier, and I. J. ten Berge. 2004. Emergence of a CD4+CD28- granzyme B+, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection. *Journal of immunology* 173: 1834-1841.

40. van Leeuwen, E. M., I. J. ten Berge, and R. A. van Lier. 2007. Induction and maintenance of CD8+ T cells specific for persistent viruses. *Advances in experimental medicine and biology* 590: 121-137.

41. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708-712.

42. Gillespie, G. M., M. R. Wills, V. Appay, C. O'Callaghan, M. Murphy, N. Smith, P. Sissons, S. Rowland-Jones, J. I. Bell, and P. A. Moss. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *J Virol* 74: 8140-8150.

43. Gamadia, L. E., E. B. Remmerswaal, J. F. Weel, F. Bemelman, R. A. van Lier, and I. J. Ten Berge. 2003. Primary immune responses to human CMV: a critical

role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. *Blood* 101: 2686-2692.

44. Chidrawar, S., N. Khan, W. Wei, A. McLarnon, N. Smith, L. Nayak, and P. Moss. 2009. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. *Clin Exp Immunol* 155: 423-432.

45. van Leeuwen, E. M., E. B. Remmerswaal, M. H. Heemskerk, I. J. ten Berge, and R. A. van Lier. 2006. Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. *Blood* 108: 3121-3127.

46. van Lier, R. A., I. J. ten Berge, and L. E. Gamadia. 2003. Human CD8(+) T-cell differentiation in response to viruses. *Nature reviews. Immunology* 3: 931-939.

47. Snyder, C. M. 2011. Buffered memory: a hypothesis for the maintenance of functional, virus-specific CD8(+) T cells during cytomegalovirus infection. *Immunologic research* 51: 195-204.

48. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *The Journal of experimental medicine* 188: 2205-2213.

49. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J Virol* 78: 5535-5545.

50. Snyder, C. M., A. Loewendorf, E. L. Bonnett, M. Croft, C. A. Benedict, and A. B. Hill. 2009. CD4+ T cell help has an epitope-dependent impact on CD8+ T cell memory inflation during murine cytomegalovirus infection. *Journal of immunology* 183: 3932-3941.

51. Walton, S. M., N. Torti, S. Mandaric, and A. Oxenius. 2011. T-cell help permits memory CD8(+) T-cell inflation during cytomegalovirus latency. *European journal of immunology* 41: 2248-2259.

52. Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, J. A. Nelson, and L. J. Picker. 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *The Journal of experimental medicine* 202: 673-685.

53. Winston, D. J., W. G. Ho, K. Bartoni, C. Du Mond, D. F. Ebeling, W. C. Buhles, and R. E. Champlin. 1993. Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients. Results of a placebo-controlled, double-blind trial. *Annals of internal medicine* 118: 179-184.

54. Zaia, J., L. Baden, M. J. Boeckh, S. Chakrabarti, H. Einsele, P. Ljungman, G. B. McDonald, H. Hirsch, B. Center for International, R. Marrow Transplant, P. National Marrow Donor, B. European, G. Marrow Transplant, B. American Society of, T. Marrow, B. Canadian, G. Marrow Transplant, A. Infectious Disease Society of, A. Society for Healthcare Epidemiology of, M. Association of Medical, C. Infectious Diseases, C. Centers for Disease, and Prevention. 2009. Viral disease prevention after hematopoietic cell transplantation. *Bone Marrow Transplant* 44: 471-482.

55. Krol, L., J. Stuchly, P. Hubacek, P. Keslova, P. Sedlacek, J. Stary, O. Hrusak, and T. Kalina. 2011. Signature profiles of CMV-specific T-cells in patients with CMV reactivation after hematopoietic SCT. *Bone Marrow Transplant* 46: 1089-1098.

56. Dolstra, H., F. Preijers, E. Van de Wiel-van Kemenade, A. Schattenberg, J. Galama, and T. de Witte. 1995. Expansion of CD8+CD57+ T cells after allogeneic BMT is related with a low incidence of relapse and with cytomegalovirus infection. *British journal of haematology* 90: 300-307.

57. Tuthill, M., F. Chen, S. Paston, H. De La Pena, S. Rusakiewicz, and A. Madrigal. 2009. The prevention and treatment of cytomegalovirus infection in haematopoietic stem cell transplantation. *Cancer Immunol Immunother* 58: 1481-1488.

58. Moss, P., and N. Khan. 2004. CD8(+) T-cell immunity to cytomegalovirus. *Hum Immunol* 65: 456-464.

59. Gratama, J. W., J. W. van Esser, C. H. Lamers, C. Tournay, B. Lowenberg, R. L. Bolhuis, and J. J. Cornelissen. 2001. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood* 98: 1358-1364.

60. Cwynarski, K., J. Ainsworth, M. Cobbold, S. Wagner, P. Mahendra, J. Apperley, J. Goldman, C. Craddock, and P. A. Moss. 2001. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood* 97: 1232-1240.

61. Bitmansour, A. D., S. L. Waldrop, C. J. Pitcher, E. Khatamzas, F. Kern, V. C. Maino, and L. J. Picker. 2001. Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus. *Journal of immunology* 167: 1151-1163.

62. Gamadia, L. E., R. J. Rentenaar, R. A. van Lier, and I. J. ten Berge. 2004. Properties of CD4(+) T cells in human cytomegalovirus infection. *Hum Immunol* 65: 486-492.

63. Foster, A. E., D. J. Gottlieb, M. Sartor, M. S. Hertzberg, and K. F. Bradstock. 2002. Cytomegalovirus-specific CD4+ and CD8+ T-cells follow a similar reconstitution pattern after allogeneic stem cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 8: 501-511.

64. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290: 92-97.

65. Pourgheysari, B., K. P. Piper, A. McLarnon, J. Arrazi, R. Bruton, F. Clark, M. Cook, P. Mahendra, C. Craddock, and P. A. Moss. 2009. Early reconstitution of effector memory CD4+ CMV-specific T cells protects against CMV reactivation following allogeneic SCT. *Bone Marrow Transplant* 43: 853-861.

66. Schulenburg, A., T. Watkins-Riedel, H. T. Greinix, W. Rabitsch, H. Loidolt, F. Keil, M. Mitterbauer, and P. Kalhs. 2001. CMV monitoring after peripheral blood stem cell and bone marrow transplantation by pp65 antigen and quantitative PCR. *Bone Marrow Transplant* 28: 765-768.

67. Kollman, C., C. W. Howe, C. Anasetti, J. H. Antin, S. M. Davies, A. H. Filipovich, J. Hegland, N. Kamani, N. A. Kernan, R. King, V. Ratanatharathorn, D. Weisdorf,

and D. L. Confer. 2001. Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood* 98: 2043-2051.

68. Jaskula, E., J. Bochenska, E. Kocwin, A. Tarnowska, and A. Lange. 2012. CMV Serostatus of Donor-Recipient Pairs Influences the Risk of CMV Infection/Reactivation in HSCT Patients. *Bone marrow research* 2012: 375075.

69. Bowden, R. A., M. Sayers, N. Flournoy, B. Newton, M. Banaji, E. D. Thomas, and J. D. Meyers. 1986. Cytomegalovirus immune globulin and seronegative blood products to prevent primary cytomegalovirus infection after marrow transplantation. *N Engl J Med* 314: 1006-1010.

70. Nichols, W. G., M. Boeckh, R. A. Carter, A. Wald, and L. Corey. 2003. Transferred herpes simplex virus immunity after stem-cell transplantation: clinical implications. *The Journal of infectious diseases* 187: 801-808.

71. Lilleri, D., G. Gerna, P. Zelini, A. Chiesa, V. Rognoni, A. Mastronuzzi, G. Giorgiani, M. Zecca, and F. Locatelli. 2012. Monitoring of human cytomegalovirus and virus-specific T-cell response in young patients receiving allogeneic hematopoietic stem cell transplantation. *PloS one* 7: e41648.

72. Lacey, S. F., G. Gallez-Hawkins, M. Crooks, J. Martinez, D. Senitzer, S. J. Forman, R. Spielberger, J. A. Zaia, and D. J. Diamond. 2002. Characterization of cytotoxic function of CMV-pp65-specific CD8+ T-lymphocytes identified by HLA tetramers in recipients and donors of stem-cell transplants. *Transplantation* 74: 722-732.

73. Ljungman, P., R. Brand, H. Einsele, F. Frassoni, D. Niederwieser, and C. Cordonnier. 2003. Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. *Blood* 102: 4255-4260.

74. Geginat, J., F. Sallusto, and A. Lanzavecchia. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *The Journal of experimental medicine* 194: 1711-1719.

75. Zhou, W., J. Longmate, S. F. Lacey, J. M. Palmer, G. Gallez-Hawkins, L. Thao, R. Spielberger, R. Nakamura, S. J. Forman, J. A. Zaia, and D. J. Diamond. 2009. Impact of donor CMV status on viral infection and reconstitution of multifunction CMV-specific T cells in CMV-positive transplant recipients. *Blood* 113: 6465-6476.

76. Nichols, W. G., L. Corey, T. Gooley, C. Davis, and M. Boeckh. 2002. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. *The Journal of infectious diseases* 185: 273-282.

77. Borchers, S., M. Bremm, T. Lehrnbecher, E. Dammann, B. Pabst, B. Wolk, R. Esser, M. Yildiz, M. Eder, M. Stadler, P. Bader, H. Martin, A. Jarisch, G. Schneider, T. Klingebiel, A. Ganser, E. M. Weissinger, and U. Koehl. 2012. Sequential anti-cytomegalovirus response monitoring may allow prediction of cytomegalovirus reactivation after allogeneic stem cell transplantation. *PloS one* 7: e50248.

78. Borchers, S., S. Luther, U. Lips, N. Hahn, J. Kontsendorn, M. Stadler, S. Buchholz, H. Diedrich, M. Eder, U. Koehl, A. Ganser, and E. Mischak-Weissinger. 2011. Tetramer monitoring to assess risk factors for recurrent cytomegalovirus reactivation and reconstitution of antiviral immunity post allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis* 13: 222-236.

79. Mead, A. J., K. J. Thomson, E. C. Morris, S. Mohamedbhai, S. Denovan, G. Orti, A. K. Fielding, P. D. Kottaridis, R. Hough, R. Chakraverty, D. C. Linch, S. Mackinnon, and K. S. Peggs. 2010. HLA-mismatched unrelated donors are a viable alternate graft source for allogeneic transplantation following alemtuzumab-based reduced-intensity conditioning. *Blood* 115: 5147-5153.

80. Hambach, L., M. Stadler, E. Dammann, A. Ganser, and B. Hertenstein. 2002. Increased risk of complicated CMV infection with the use of mycophenolate mofetil in allogeneic stem cell transplantation. *Bone Marrow Transplant* 29: 903-906.

81. Emery, V. C., C. A. Sabin, A. V. Cope, D. Gor, A. F. Hassan-Walker, and P. D. Griffiths. 2000. Application of viral-load kinetics to identify patients who develop cytomegalovirus disease after transplantation. *Lancet* 355: 2032-2036.

82. Nichols, W. G., L. Corey, T. Gooley, W. L. Drew, R. Miner, M. Huang, C. Davis, and M. Boeckh. 2001. Rising pp65 antigenemia during preemptive anticytomegalovirus therapy after allogeneic hematopoietic stem cell transplantation: risk factors, correlation with DNA load, and outcomes. *Blood* 97: 867-874.

83. Nakamae, H., K. A. Kirby, B. M. Sandmaier, L. Norasetthada, D. G. Maloney, M. B. Maris, C. Davis, L. Corey, R. Storb, and M. Boeckh. 2009. Effect of conditioning regimen intensity on CMV infection in allogeneic hematopoietic cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 15: 694-703.

84. Kim, S. H., S. Y. Kee, D. G. Lee, S. M. Choi, S. H. Park, J. C. Kwon, K. S. Eom, Y. J. Kim, H. J. Kim, S. Lee, C. K. Min, D. W. Kim, J. H. Choi, J. H. Yoo, J. W. Lee, and W. S. Min. 2013. Infectious complications following allogeneic stem cell transplantation: reduced-intensity vs. myeloablative conditioning regimens. *Transpl Infect Dis* 15: 49-59.

85. Kanda, Y., K. Oshima, S. Kako, T. Fukuda, N. Uchida, K. Miyamura, Y. Kondo, S. Nakao, K. Nagafuji, T. Miyamoto, M. Kurokawa, Y. Okoshi, S. Chiba, Y. Ohashi, Y. Takaue, and S. Taniguchi. 2013. In vivo T-cell depletion with alemtuzumab in allogeneic hematopoietic stem cell transplantation: Combined results of two studies on aplastic anemia and HLA-mismatched haploidentical transplantation. *Am J Hematol* 88: 294-300.

86. Lilleri, D., G. Gerna, C. Fornara, A. Chiesa, G. Comolli, M. Zecca, and F. Locatelli. 2009. Human cytomegalovirus-specific T cell reconstitution in young patients receiving T cell-depleted, allogeneic hematopoietic stem cell transplantation. *The Journal of infectious diseases* 199: 829-836.

87. Perez-Martinez, A., M. Gonzalez-Vicent, J. Valentin, E. Aleo, A. Lassaletta, J. Sevilla, J. L. Vicario, M. Ramirez, and M. A. Diaz. 2012. Early evaluation of immune reconstitution following allogeneic CD3/CD19-depleted grafts from

alternative donors in childhood acute leukemia. *Bone Marrow Transplant* 47: 1419-1427.

88. Lang, P., H. M. Teltschik, T. Feuchtinger, I. Muller, M. Pfeiffer, M. Schumm, M. Ebinger, C. P. Schwarze, B. Gruhn, A. Schrauder, M. H. Albert, J. Greil, C. Urban, and R. Handgretinger. 2014. Transplantation of CD3/CD19 depleted allografts from haploidentical family donors in paediatric leukaemia. *British journal of haematology* 165: 688-698.

89. Federmann, B., M. Hagele, M. Pfeiffer, S. Wirths, M. Schumm, C. Faul, W. Vogel, R. Handgretinger, L. Kanz, and W. A. Bethge. 2010. Immune reconstitution after haploidentical hematopoietic cell transplantation: impact of reduced intensity conditioning and CD3/CD19 depleted grafts. *Leukemia* 25: 121-129.

90. Airoldi, I., A. Bertaina, I. Prigione, A. Zorzoli, D. Pagliara, C. Cocco, R. Meazza, F. Loiacono, B. Lucarelli, M. E. Bernardo, G. Barbarito, D. Pende, A. Moretta, V. Pistoia, L. Moretta, and F. Locatelli. 2015. gammadelta T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-alphabeta+/CD19+ lymphocytes. *Blood* 125: 2349-2358.

91. Ruutu, T., P. Ljungman, L. Brinch, S. Lenhoff, B. Lonnqvist, O. Ringden, P. Ruutu, L. Volin, D. Albrechtsen, B. Sallerfors, F. Ebeling, and G. Myllyla. 1997. No prevention of cytomegalovirus infection by anti-cytomegalovirus hyperimmune globulin in seronegative bone marrow transplant recipients. The Nordic BMT Group. *Bone Marrow Transplant* 19: 233-236.

92. Ljungman, P., R. de La Camara, N. Milpied, L. Volin, C. A. Russell, A. Crisp, and A. Webster. 2002. Randomized study of valacyclovir as prophylaxis against cytomegalovirus reactivation in recipients of allogeneic bone marrow transplants. *Blood* 99: 3050-3056.

93. Venton, G., R. Crocchiolo, S. Furst, A. Granata, C. Oudin, C. Faucher, D. Coso, R. Bouabdallah, P. Berger, N. Vey, P. Ladaique, C. Chabannon, M. le Merlin, D. Blaise, and J. El-Cheikh. 2014. Risk factors of Ganciclovir-related neutropenia after allogeneic stem cell transplantation: a retrospective monocentre study on 547 patients. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 20: 160-166.

94. Hebart, H., S. Daginik, S. Stevanovic, U. Grigoleit, A. Dobler, M. Baur, G. Rauser, C. Sinzger, G. Jahn, J. Loeffler, L. Kanz, H. G. Rammensee, and H. Einsele. 2002. Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon-gamma-enzyme-linked immunospot assay and flow cytometry in healthy individuals and in patients after allogeneic stem cell transplantation. *Blood* 99: 3830-3837.

95. Li, C. R., P. D. Greenberg, M. J. Gilbert, J. M. Goodrich, and S. R. Riddell. 1994. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. *Blood* 83: 1971-1979.

96. Boeckh, M., T. A. Gooley, D. Myerson, T. Cunningham, G. Schoch, and R. A. Bowden. 1996. Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir versus ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study. *Blood* 88: 4063-4071.

97. Vij, R., H. Khoury, R. Brown, L. T. Goodnough, S. M. Devine, W. Blum, D. Adkins, and J. F. DiPersio. 2003. Low-dose short-course intravenous ganciclovir as pre-emptive therapy for CMV viremia post allo-PBSC transplantation. *Bone Marrow Transplant* 32: 703-707.

98. Mori, T., Y. Aisa, T. Nakazato, Y. Ikeda, and S. Okamoto. 2005. Risk-adapted preemptive therapy for cytomegalovirus diseases after allogeneic hematopoietic stem cell transplantation. *International journal of hematology* 81: 439-440.

99. Pescovitz, M. D., J. Rabkin, R. M. Merion, C. V. Paya, J. Pirsch, R. B. Freeman, J. O'Grady, C. Robinson, Z. To, K. Wren, L. Banken, W. Buhles, and F. Brown. 2000. Valganciclovir results in improved oral absorption of ganciclovir in liver transplant recipients. *Antimicrob Agents Chemother* 44: 2811-2815.

100. Andrei, G., E. De Clercq, and R. Snoeck. 2008. Novel inhibitors of human CMV. *Current opinion in investigational drugs* 9: 132-145.

101. Reusser, P., H. Einsele, J. Lee, L. Volin, M. Rovira, D. Engelhard, J. Finke, C. Cordonnier, H. Link, and P. Ljungman. 2002. Randomized multicenter trial of foscarnet versus ganciclovir for preemptive therapy of cytomegalovirus infection after allogeneic stem cell transplantation. *Blood* 99: 1159-1164.

102. Oshima, K., Y. Kanda, S. Kako, Y. Asano-Mori, T. Watanabe, T. Motokura, S. Chiba, K. Shiraki, and M. Kurokawa. 2008. Case report: persistent cytomegalovirus (CMV) infection after haploidentical hematopoietic stem cell transplantation using in vivo alemtuzumab: emergence of resistant CMV due to mutations in the UL97 and UL54 genes. *J Med Virol* 80: 1769-1775.

103. Winston, D. J., J. A. Young, V. Pullarkat, G. A. Papanicolaou, R. Vij, E. Vance, G. J. Alangaden, R. F. Chemaly, F. Petersen, N. Chao, J. Klein, K. Sprague, S. A. Villano, and M. Boeckh. 2008. Maribavir prophylaxis for prevention of cytomegalovirus infection in allogeneic stem cell transplant recipients: a multicenter, randomized, double-blind, placebo-controlled, dose-ranging study. *Blood* 111: 5403-5410.

104. Marty, F. M., P. Ljungman, G. A. Papanicolaou, D. J. Winston, R. F. Chemaly, L. Strasfeld, J. A. Young, T. Rodriguez, J. Maertens, M. Schmitt, H. Einsele, A. Ferrant, J. H. Lipton, S. A. Villano, H. Chen, M. Boeckh, and G. Maribavir -300 Clinical Study. 2011. Maribavir prophylaxis for prevention of cytomegalovirus disease in recipients of allogeneic stem-cell transplants: a phase 3, double-blind, placebo-controlled, randomised trial. *The Lancet. Infectious diseases* 11: 284-292.

105. Marty, F. M., D. J. Winston, S. D. Rowley, E. Vance, G. A. Papanicolaou, K. M. Mullane, T. M. Brundage, A. T. Robertson, S. Godkin, H. Mommeja-Marin, M. Boeckh, and C. M. X. C. S. Group. 2013. CMX001 to prevent cytomegalovirus disease in hematopoietic-cell transplantation. *N Engl J Med* 369: 1227-1236.

106. Chemaly, R. F., A. J. Ullmann, S. Stoelben, M. P. Richard, M. Bornhauser, C. Groth, H. Einsele, M. Silverman, K. M. Mullane, J. Brown, H. Nowak, K. Kolling, H. P. Stobernack, P. Lischka, H. Zimmermann, H. Rubsamen-Schaeff, R. E. Champlin, G. Ehninger, and A. I. C. S. Team. 2014. Letermovir for cytomegalovirus prophylaxis in hematopoietic-cell transplantation. *N Engl J Med* 370: 1781-1789.

107. Martin, D. F., J. Sierra-Madero, S. Walmsley, R. A. Wolitz, K. Macey, P. Georgiou, C. A. Robinson, M. J. Stempien, and G. Valganciclovir Study. 2002. A

controlled trial of valganciclovir as induction therapy for cytomegalovirus retinitis. *N Engl J Med* 346: 1119-1126.

108. Busca, A., P. de Fabritiis, V. Ghisetti, T. Allice, M. Mirabile, G. Gentile, F. Locatelli, and M. Falda. 2007. Oral valganciclovir as preemptive therapy for cytomegalovirus infection post allogeneic stem cell transplantation. *Transpl Infect Dis* 9: 102-107.

109. Boeckh, M., W. G. Nichols, G. Papanicolaou, R. Rubin, J. R. Wingard, and J. Zaia. 2003. Cytomegalovirus in hematopoietic stem cell transplant recipients: Current status, known challenges, and future strategies. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 9: 543-558.

110. Hebart, H., and H. Einsele. 2004. Clinical aspects of CMV infection after stem cell transplantation. *Hum Immunol* 65: 432-436.

111. Ozdemir, E., R. M. Saliba, R. E. Champlin, D. R. Couriel, S. A. Giralt, M. de Lima, I. F. Khouri, C. Hosing, S. M. Kornblau, P. Anderlini, E. J. Shpall, M. H. Qazilbash, J. J. Molldrem, R. F. Chemaly, and K. V. Komanduri. 2007. Risk factors associated with late cytomegalovirus reactivation after allogeneic stem cell transplantation for hematological malignancies. *Bone Marrow Transplant* 40: 125-136.

112. Mercorelli, B., E. Sinigalia, A. Loregian, and G. Palu. 2008. Human cytomegalovirus DNA replication: antiviral targets and drugs. *Reviews in medical virology* 18: 177-210.

113. Einsele, H., H. Hebart, C. Kauffmann-Schneider, C. Sinzger, G. Jahn, P. Bader, T. Klingebiel, K. Dietz, J. Loffler, C. Bokemeyer, C. A. Muller, and L. Kanz. 2000. Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. *Bone Marrow Transplant* 25: 757-763.

114. Krause, H., H. Hebart, G. Jahn, C. A. Muller, and H. Einsele. 1997. Screening for CMV-specific T cell proliferation to identify patients at risk of developing late onset CMV disease. *Bone Marrow Transplant* 19: 1111-1116.

115. Bollard, C. M., S. Gottschalk, A. M. Leen, H. Weiss, K. C. Straathof, G. Carrum, M. Khalil, M. F. Wu, M. H. Huls, C. C. Chang, M. V. Gresik, A. P. Gee, M. K. Brenner, C. M. Rooney, and H. E. Heslop. 2007. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood* 110: 2838-2845.

116. Riddell, S. R., B. A. Walter, M. J. Gilbert, and P. D. Greenberg. 1994. Selective reconstitution of CD8+ cytotoxic T lymphocyte responses in immunodeficient bone marrow transplant recipients by the adoptive transfer of T cell clones. *Bone Marrow Transplant* 14 Suppl 4: S78-84.

117. Feuchtinger, T., K. Opherk, W. A. Bethge, M. S. Topp, F. R. Schuster, E. M. Weissinger, M. Mohty, R. Or, M. Maschan, M. Schumm, K. Hamprecht, R. Handgretinger, P. Lang, and H. Einsele. 2010. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood* 116: 4360-4367.

118. Peggs, K. S. 2009. Adoptive T cell immunotherapy for cytomegalovirus. *Expert Opin Biol Ther* 9: 725-736.

119. Cobbold, M., N. Khan, B. Pourgheysari, S. Tauro, D. McDonald, H. Osman, M. Assenmacher, L. Billingham, C. Steward, C. Crawley, E. Olavarria, J. Goldman, R. Chakraverty, P. Mahendra, C. Craddock, and P. A. Moss. 2005. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *The Journal of experimental medicine* 202: 379-386.

120. Peggs, K. S., K. Thomson, E. Samuel, G. Dyer, J. Armoogum, R. Chakraverty, K. Pang, S. Mackinnon, and M. W. Lowdell. 2011. Directly Selected Cytomegalovirus-Reactive Donor T Cells Confer Rapid and Safe Systemic Reconstitution of Virus-Specific Immunity Following Stem Cell Transplantation. *Clin Infect Dis* 52: 49-57.

121. Leen, A. M., G. D. Myers, U. Sili, M. H. Huls, H. Weiss, K. S. Leung, G. Carrum, R. A. Krance, C. C. Chang, J. J. Molldrem, A. P. Gee, M. K. Brenner, H. E. Heslop, C. M. Rooney, and C. M. Bollard. 2006. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med* 12: 1160-1166.

122. Gerdemann, U., J. M. Keirnan, U. L. Katari, R. Yanagisawa, A. S. Christin, L. E. Huye, S. K. Perna, S. Ennamuri, S. Gottschalk, M. K. Brenner, H. E. Heslop, C. M. Rooney, and A. M. Leen. 2012. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. *Molecular therapy : the journal of the American Society of Gene Therapy* 20: 1622-1632.

123. Hanley, P. J., C. R. Cruz, B. Savoldo, A. M. Leen, M. Stanojevic, M. Khalil, W. Decker, J. J. Molldrem, H. Liu, A. P. Gee, C. M. Rooney, H. E. Heslop, G. Dotti, M. K. Brenner, E. J. Shpall, and C. M. Bollard. 2009. Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes. *Blood* 114: 1958-1967.

124. Maus, M. V., J. A. Fraietta, B. L. Levine, M. Kalos, Y. Zhao, and C. H. June. 2014. Adoptive immunotherapy for cancer or viruses. *Annu Rev Immunol* 32: 189-225.

125. Einsele, H., E. Roosnek, N. Rufer, C. Sinzger, S. Riegler, J. Loffler, U. Grigoleit, A. Moris, H. G. Rammensee, L. Kanz, A. Kleihauer, F. Frank, G. Jahn, and H. Hebart. 2002. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood* 99: 3916-3922.

126. Hill, G. R., S. K. Tey, L. Beagley, T. Crough, J. A. Morton, A. D. Clouston, P. Whiting, and R. Khanna. 2010. Successful immunotherapy of HCMV disease using virus-specific T cells expanded from an allogeneic stem cell transplant recipient. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 10: 173-179.

127. Blyth, E., L. Clancy, R. Simms, C. K. Ma, J. Burgess, S. Deo, K. Byth, M. C. Dubosq, P. J. Shaw, K. P. Micklethwaite, and D. J. Gottlieb. 2013. Donor-derived

CMV-specific T cells reduce the requirement for CMV-directed pharmacotherapy after allogeneic stem cell transplantation. *Blood* 121: 3745-3758.

128. Ramirez, N., L. Beloki, M. Ciaurriz, M. Rodriguez-Calvillo, D. Escors, C. Mansilla, E. Bandres, and E. Olavarria. 2013. Impact of T cell selection methods in the success of clinical adoptive immunotherapy. *Cell Mol Life Sci*.

129. Saglio, F., P. J. Hanley, and C. M. Bollard. 2014. The time is now: moving toward virus-specific T cells after allogeneic hematopoietic stem cell transplantation as the standard of care. *Cytotherapy* 16: 149-159.

130. Meisel, R., S. Brockers, K. Heseler, O. Degistirici, H. Bulle, C. Woite, S. Stuhlsatz, W. Schwippert, M. Jager, R. Sorg, R. Henschler, J. Seissler, D. Dilloo, and W. Daubener. 2011. Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia* 25: 648-654.

131. Meisel, R., K. Heseler, J. Nau, S. K. Schmidt, M. Leineweber, S. Pudelko, J. Wenning, A. Zimmermann, H. Hengel, C. Sinzger, O. Degistirici, R. V. Sorg, and W. Daubener. 2014. Cytomegalovirus infection impairs immunosuppressive and antimicrobial effector functions of human multipotent mesenchymal stromal cells. *Mediators of inflammation* 2014: 898630.

132. Boeckh, M., T. Stevens-Ayers, and R. A. Bowden. 1996. Cytomegalovirus pp65 antigenemia after autologous marrow and peripheral blood stem cell transplantation. *The Journal of infectious diseases* 174: 907-912.

133. Solano, C., I. Munoz, A. Gutierrez, A. Farga, F. Prosper, J. Garcia-Conde, D. Navarro, and C. Gimeno. 2001. Qualitative plasma PCR assay (AMPLICOR CMV test) versus pp65 antigenemia assay for monitoring cytomegalovirus viremia and guiding preemptive ganciclovir therapy in allogeneic stem cell transplantation. *Journal of clinical microbiology* 39: 3938-3941.

134. Mariotti, J., F. Maura, F. Spina, L. Roncari, A. Dodero, L. Farina, V. Montefusco, C. Carniti, B. Sarina, F. Patriarca, A. Rambaldi, F. Onida, A. Olivieri, F. Zallio, and P. Corradini. 2014. Impact of cytomegalovirus replication and cytomegalovirus serostatus on the outcome of patients with B cell lymphoma after allogeneic stem cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 20: 885-890.

135. Einsele, H., G. Ehninger, H. Hebart, K. M. Wittkowski, U. Schuler, G. Jahn, P. Mackes, M. Herter, T. Klingebiel, J. Loffler, S. Wagner, and C. A. Muller. 1995. Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. *Blood* 86: 2815-2820.

136. Patel, R., T. F. Smith, M. Espy, D. Portela, R. H. Wiesner, R. A. Krom, and C. V. Paya. 1995. A prospective comparison of molecular diagnostic techniques for the early detection of cytomegalovirus in liver transplant recipients. *The Journal of infectious diseases* 171: 1010-1014.

137. Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* 88: 7276-7280.

138. Marchetti, S., R. Santangelo, S. Manzara, S. D'Onghia, G. Fadda, and P. Cattani. 2011. Comparison of real-time PCR and pp65 antigen assays for monitoring the development of Cytomegalovirus disease in recipients of solid organ and bone marrow transplants. *New Microbiol* 34: 157-164.

139. Mori, T., S. Mori, Y. Kanda, K. Yakushiji, S. Mineishi, Y. Takaue, H. Gondo, M. Harada, H. Sakamaki, T. Yajima, Y. Iwao, T. Hibi, and S. Okamoto. 2004. Clinical significance of cytomegalovirus (CMV) antigenemia in the prediction and diagnosis of CMV gastrointestinal disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 33: 431-434.

140. Orii, T., N. Ohkohchi, H. Kikuchi, N. Koyamada, S. Chubachi, S. Satomi, H. Kimura, Y. Hoshino, and M. Morita. 2000. Usefulness of quantitative real-time polymerase chain reaction in following up patients with Epstein-Barr virus infection after liver transplantation. *Clinical transplantation* 14: 308-317.

141. Nomura, L., V. C. Maino, and H. T. Maecker. 2008. Standardization and optimization of multiparameter intracellular cytokine staining. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 73: 984-991.

142. Lamoreaux, L., M. Roederer, and R. Koup. 2006. Intracellular cytokine optimization and standard operating procedure. *Nature protocols* 1: 1507-1516.

143. Borchers, S., J. Ogonek, P. R. Varanasi, S. Tischer, M. Bremm, B. Eiz-Vesper, U. Koehl, and E. M. Weissinger. 2013. Multimer monitoring of CMV-specific T cells in research and in clinical applications. *Diagn Microbiol Infect Dis*.

144. Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-Jones, and A. D. Kelleher. 2002. Characterization of CD4(+) CTLs ex vivo. *Journal of immunology* 168: 5954-5958.

145. Abu-Khader, A., and S. Krause. 2013. Rapid monitoring of immune reconstitution after allogeneic stem cell transplantation--a comparison of different assays for the detection of cytomegalovirus-specific T cells. *European journal of haematology* 91: 534-545.

146. Letsch, A., and C. Scheibenbogen. 2003. Quantification and characterization of specific T-cells by antigen-specific cytokine production using ELISPOT assay or intracellular cytokine staining. *Methods* 31: 143-149.

147. Letsch, A., U. Keilholz, G. Assfalg, V. Mailander, E. Thiel, and C. Scheibenbogen. 2003. Bone marrow contains melanoma-reactive CD8+ effector T cells and, compared with peripheral blood, enriched numbers of melanoma-reactive CD8+ memory T cells. *Cancer research* 63: 5582-5586.

148. Hobeika, A. C., M. A. Morse, T. Osada, M. Ghanayem, D. Niedzwiecki, R. Barrier, H. K. Lyerly, and T. M. Clay. 2005. Enumerating antigen-specific T-cell responses in peripheral blood: a comparison of peptide MHC Tetramer, ELISpot, and intracellular cytokine analysis. *Journal of immunotherapy* 28: 63-72.

149. Ohnishi, M., T. Sakurai, Y. Heike, R. Yamazaki, Y. Kanda, Y. Takaue, H. Mizoguchi, and Y. Kawakami. 2005. Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic haematopoietic stem cell transplantation using interferon-gamma-enzyme-linked immunospot and human

leucocyte antigen tetramer assays with an immunodominant T-cell epitope. *British journal of haematology* 131: 472-479.

150. Lehmann, P. V., and W. Zhang. 2012. Unique strengths of ELISPOT for T cell diagnostics. *Methods in molecular biology* 792: 3-23.

151. Tischer, S., D. Dieks, C. Sukdolak, C. Bunse, C. Figueiredo, S. Immenschuh, S. Borchers, R. Stripecke, B. Maecker-Kolhoff, R. Blasczyk, and B. Eiz-Vesper. 2014. Evaluation of suitable target antigens and immunoassays for high-accuracy immune monitoring of cytomegalovirus and Epstein-Barr virus-specific T cells as targets of interest in immunotherapeutic approaches. *J Immunol Methods* 408: 101-113.

152. Thiel, A., A. Scheffold, and A. Radbruch. 2004. Antigen-specific cytometry--new tools arrived! *Clin Immunol* 111: 155-161.

153. Barron, M. A., D. Gao, K. L. Springer, J. A. Patterson, M. W. Brunvand, P. A. McSweeney, C. Zeng, A. E. Baron, and A. Weinberg. 2009. Relationship of reconstituted adaptive and innate cytomegalovirus (CMV)-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 49: 1777-1783.

154. Sukdolak, C., S. Tischer, D. Dieks, C. Figueiredo, L. Goudeva, H. G. Heuft, M. Verboom, S. Immenschuh, A. Heim, S. Borchers, E. Mischak-Weissinger, R. Blasczyk, B. Maecker-Kolhoff, and B. Eiz-Vesper. 2013. CMV-, EBV- and ADV-specific T cell immunity: screening and monitoring of potential third-party donors to improve post-transplantation outcome. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 19: 1480-1492.

155. Casalegno-Garduno, R., A. Schmitt, J. Yao, X. Wang, X. Xu, M. Freund, and M. Schmitt. 2010. Multimer technologies for detection and adoptive transfer of antigen-specific T cells. *Cancer Immunol Immunother* 59: 195-202.

156. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94-96.

157. Gratama, J. W., M. Boeckh, R. Nakamura, J. J. Cornelissen, R. A. Brooimans, J. A. Zaia, S. J. Forman, K. Gaal, K. R. Bray, G. H. Gasior, C. S. Boyce, L. A. Sullivan, and P. C. Southwick. 2010. Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. *Blood* 116: 1655-1662.

158. Koehl, U., E. Dirkwinkel, M. Koenig, S. Erben, J. Soerensen, P. Bader, H. W. Doerr, W. Preiser, E. Weissinger, T. Klingebiel, H. Martin, and T. Lehrnbecher. 2008. Reconstitution of cytomegalovirus specific T cells after pediatric allogeneic stem cell transplantation: results from a pilot study using a multi-allele CMV tetramer group. *Klinische Padiatrie* 220: 348-352.

159. Duplan, V., E. Suberbielle, C. E. Napper, E. Joly, A. Saoudi, and D. Gonzalez-Dunia. 2007. Tracking antigen-specific CD8+ T cells in the rat using MHC class I multimers. *J Immunol Methods* 320: 30-39.

160. Lee, S., J. B. Park, E. Y. Kim, S. Y. Joo, E. C. Shin, C. H. Kwon, J. W. Joh, and S. J. Kim. 2011. Monitoring of cytomegalovirus-specific CD8+ T-cell response with major histocompatibility complex pentamers in kidney transplant recipients. *Transplant Proc* 43: 2636-2640.

161. Knabel, M., T. J. Franz, M. Schiemann, A. Wulf, B. Villmow, B. Schmidt, H. Bernhard, H. Wagner, and D. H. Busch. 2002. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med* 8: 631-637.

162. Tischer, S., T. Kaireit, C. Figueiredo, O. Hiller, B. Maecker-Kolhoff, R. Geyeregger, S. Immenschuh, R. Blasczyk, and B. Eiz-Vesper. 2012. Establishment of the reversible peptide-major histocompatibility complex (pMHC) class I Histamer technology: tool for visualization and selection of functionally active antigen-specific CD8(+) T lymphocytes. *Int Immunol* 24: 561-572.

163. Neudorfer, J., B. Schmidt, K. M. Huster, F. Anderl, M. Schiemann, G. Holzapfel, T. Schmidt, L. Germeroth, H. Wagner, C. Peschel, D. H. Busch, and H. Bernhard. 2007. Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* 320: 119-131.

164. Hadrup, S. R., A. H. Bakker, C. J. Shu, R. S. Andersen, J. van Veluw, P. Hombrink, E. Castermans, P. Thor Straten, C. Blank, J. B. Haanen, M. H. Heemskerk, and T. N. Schumacher. 2009. Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Methods* 6: 520-526.

165. Andersen, R. S., P. Kvistborg, T. M. Frosig, N. W. Pedersen, R. Lyngaa, A. H. Bakker, C. J. Shu, P. Straten, T. N. Schumacher, and S. R. Hadrup. 2012. Parallel detection of antigen-specific T cell responses by combinatorial encoding of MHC multimers. *Nature protocols* 7: 891-902.

166. Vollers, S. S., and L. J. Stern. 2008. Class II major histocompatibility complex tetramer staining: progress, problems, and prospects. *Immunology* 123: 305-313.

167. Cole, D. K., N. J. Pumphrey, J. M. Boulter, M. Sami, J. I. Bell, E. Gostick, D. A. Price, G. F. Gao, A. K. Sewell, and B. K. Jakobsen. 2007. Human TCR-binding affinity is governed by MHC class restriction. *Journal of immunology* 178: 5727-5734.

168. Nepom, G. T. 2012. MHC class II tetramers. *Journal of immunology* 188: 2477-2482.

169. Kwok, W. W., V. Tan, L. Gillette, C. T. Littell, M. A. Soltis, R. B. LaFond, J. Yang, E. A. James, and J. H. DeLong. 2012. Frequency of epitope-specific naive CD4(+) T cells correlates with immunodominance in the human memory repertoire. *Journal of immunology* 188: 2537-2544.

170. Scriba, T. J., M. Purbhoo, C. L. Day, N. Robinson, S. Fidler, J. Fox, J. N. Weber, P. Klenerman, A. K. Sewell, and R. E. Phillips. 2005. Ultrasensitive detection and phenotyping of CD4+ T cells with optimized HLA class II tetramer staining. *Journal of immunology* 175: 6334-6343.

171. Harcourt, G. C., T. J. Scriba, N. Semmo, S. Bounds, E. Taylor, and P. Klenerman. 2006. Identification of key peptide-specific CD4+ T cell responses to human cytomegalovirus: implications for tracking antiviral populations. *Clin Exp Immunol* 146: 203-210.

172. Ayyoub, M., D. Dojcinovic, P. Pignon, I. Raimbaud, J. Schmidt, I. Luescher, and D. Valmori. 2010. Monitoring of NY-ESO-1 specific CD4+ T cells using molecularly defined MHC class II/His-tag-peptide tetramers. *Proceedings of the National Academy of Sciences of the United States of America* 107: 7437-7442.

173. Braendstrup, P., S. Justesen, T. Osterbye, L. L. Nielsen, R. Mallone, L. Vindelov, A. Stryhn, and S. Buus. 2013. MHC class II tetramers made from isolated recombinant alpha and beta chains refolded with affinity-tagged peptides. *PloS one* 8: e73648.

174. Beloki, L., M. Ciaurriz, C. Mansilla, E. Bandres, M. Rodriguez-Calvillo, N. Ramirez, and E. Olavarria. 2013. The abrogation of TCR-independent interactions with human serum ensures a selective capture of therapeutic virus-specific CD8 T-cells by Multimer Technology in Adoptive Immunotherapy. *J Immunol Methods*.

175. Bunce, M., C. M. O'Neill, M. C. Barnardo, P. Krausa, M. J. Browning, P. J. Morris, and K. I. Welsh. 1995. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* 46: 355-367.

176. Brown, R. L., R. L. Griffith, R. H. Neubauer, and H. Rabin. 1983. Development of a serum-free medium which supports the long-term growth of human and nonhuman primate lymphoid cells. *Journal of cellular physiology* 115: 191-198.

177. Rabin, H., R. F. Hopkins, 3rd, F. W. Ruscetti, R. H. Neubauer, R. L. Brown, and T. G. Kawakami. 1981. Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumor T cells. *Journal of immunology* 127: 1852-1856.

178. Ghanekar, S. A., L. E. Nomura, M. A. Suni, L. J. Picker, H. T. Maecker, and V. C. Maino. 2001. Gamma interferon expression in CD8(+) T cells is a marker for circulating cytotoxic T lymphocytes that recognize an HLA A2-restricted epitope of human cytomegalovirus phosphoprotein pp65. *Clinical and diagnostic laboratory immunology* 8: 628-631.

179. Welten, S. P., A. Redeker, K. L. Franken, C. A. Benedict, H. Yagita, F. M. Wensveen, J. Borst, C. J. Melief, R. A. van Lier, K. P. van Gisbergen, and R. Arens. 2013. CD27-CD70 costimulation controls T cell immunity during acute and persistent cytomegalovirus infection. *J Virol* 87: 6851-6865.

180. Saez-Borderias, A., M. Guma, A. Angulo, B. Bellosillo, D. Pende, and M. Lopez-Botet. 2006. Expression and function of NKG2D in CD4+ T cells specific for human cytomegalovirus. *European journal of immunology* 36: 3198-3206.

181. Alonso-Arias, R., M. A. Moro-Garcia, A. Lopez-Vazquez, L. Rodrigo, J. Baltar, F. M. Garcia, J. J. Jaurrieta, and C. Lopez-Larrea. 2011. NKG2D expression in CD4+ T lymphocytes as a marker of senescence in the aged immune system. *Age* 33: 591-605.

182. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. CD4+CD25high regulatory cells in human peripheral blood. *Journal of immunology* 167: 1245-1253.

183. Sawai, H., Y. W. Park, X. He, J. J. Goronzy, and C. M. Weyand. 2007. Fractalkine mediates T cell-dependent proliferation of synovial fibroblasts in rheumatoid arthritis. *Arthritis and rheumatism* 56: 3215-3225.

184. Hertoghs, K. M., P. D. Moerland, A. van Stijn, E. B. Remmerswaal, S. L. Yong, P. J. van de Berg, S. M. van Ham, F. Baas, I. J. ten Berge, and R. A. van Lier. 2010. Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. *The Journal of clinical investigation* 120: 4077-4090.

185. Schmitt, A., T. Tonn, D. H. Busch, G. U. Grigoleit, H. Einsele, M. Odendahl, L. Germeroth, M. Ringhoffer, S. Ringhoffer, M. Wiesneth, J. Greiner, D. Michel, T. Mertens, M. Rojewski, M. Marx, S. von Harsdorf, H. Dohner, E. Seifried, D. Bunjes, and M. Schmitt. 2010. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion* 51: 591-599.

186. Aarntzen, E. H., I. J. De Vries, W. J. Lesterhuis, D. Schuurhuis, J. F. Jacobs, K. Bol, G. Schreibelt, R. Mus, J. H. De Wilt, J. B. Haanen, D. Schadendorf, A. Croockewit, W. A. Blokx, M. M. Van Rossum, W. W. Kwok, G. J. Adema, C. J. Punt, and C. G. Figdor. 2013. Targeting CD4(+) T-helper cells improves the induction of antitumor responses in dendritic cell-based vaccination. *Cancer research* 73: 19-29.

187. Bodinier, M., M. A. Peyrat, C. Tournay, F. Davodeau, F. Romagne, M. Bonneville, and F. Lang. 2000. Efficient detection and immunomagnetic sorting of specific T cells using multimers of MHC class I and peptide with reduced CD8 binding. *Nat Med* 6: 707-710.

188. Cole, D. K., B. Laugel, M. Clement, D. A. Price, L. Wooldridge, and A. K. Sewell. 2012. The molecular determinants of CD8 co-receptor function. *Immunology* 137: 139-148.

189. Wooldridge, L., A. Lissina, J. Vernazza, E. Gostick, B. Laugel, S. L. Hutchinson, F. Mirza, P. R. Dunbar, J. M. Boulter, M. Glick, V. Cerundolo, H. A. van den Berg, D. A. Price, and A. K. Sewell. 2007. Enhanced immunogenicity of CTL antigens through mutation of the CD8 binding MHC class I invariant region. *European journal of immunology* 37: 1323-1333.

190. Dolton, G., A. Lissina, A. Skowera, K. Ladell, K. Tungatt, E. Jones, D. Kronenberg-Versteeg, H. Akpovwa, J. M. Pentier, C. J. Holland, A. J. Godkin, D. K. Cole, M. A. Neller, J. J. Miles, D. A. Price, M. Peakman, and A. K. Sewell. 2014. Comparison of peptide-major histocompatibility complex tetramers and dextramers for the identification of antigen-specific T cells. *Clin Exp Immunol* 177: 47-63.

191. Guillaume, P., D. F. Legler, N. Boucheron, M. A. Doucey, J. C. Cerottini, and I. F. Luescher. 2003. Soluble major histocompatibility complex-peptide octamers with impaired CD8 binding selectively induce Fas-dependent apoptosis. *J Biol Chem* 278: 4500-4509.

192. Beloki, L., M. Ciaurriz, C. Mansilla, A. Zabalza, E. Perez-Valderrama, E. R. Samuel, M. W. Lowdell, N. Ramirez, and E. Olavarria. 2015. Assessment of the effector function of CMV-specific CTLs isolated using MHC-multimers from granulocyte-colony stimulating factor mobilized peripheral blood. *Journal of translational medicine* 13: 165.

193. Newell, E. W., L. O. Klein, W. Yu, and M. M. Davis. 2009. Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. *Nat Methods* 6: 497-499.

194. Storek, J., T. Gooley, R. P. Witherspoon, K. M. Sullivan, and R. Storb. 1997. Infectious morbidity in long-term survivors of allogeneic marrow transplantation is associated with low CD4 T cell counts. *Am J Hematol* 54: 131-138.

195. Boeckh, M., W. Leisenring, S. R. Riddell, R. A. Bowden, M. L. Huang, D. Myerson, T. Stevens-Ayers, M. E. Flowers, T. Cunningham, and L. Corey. 2003. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. *Blood* 101: 407-414.

196. Hakki, M., S. R. Riddell, J. Storek, R. A. Carter, T. Stevens-Ayers, P. Sudour, K. White, L. Corey, and M. Boeckh. 2003. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. *Blood* 102: 3060-3067.

197. Moins-Teisserenc, H., M. Busson, C. Scieux, V. Bajzik, J. M. Cayuela, E. Clave, R. P. de Latour, F. Agbalika, P. Ribaud, M. Robin, V. Rocha, E. Gluckman, D. Charron, G. Socie, and A. Toubert. 2008. Patterns of cytomegalovirus reactivation are associated with distinct evolutive profiles of immune reconstitution after allogeneic hematopoietic stem cell transplantation. *The Journal of infectious diseases* 198: 818-826.

198. Chen, F. E., G. Aubert, P. Travers, I. A. Dodi, and J. A. Madrigal. 2002. HLA tetramers and anti-CMV immune responses: from epitope to immunotherapy. *Cytotherapy* 4: 41-48.

199. Gallez-Hawkins, G., L. Thao, S. F. Lacey, J. Martinez, X. Li, A. E. Franck, N. A. Lomeli, J. Longmate, D. J. Diamond, R. Spielberger, S. J. Forman, and J. A. Zaia. 2005. Cytomegalovirus immune reconstitution occurs in recipients of allogeneic hematopoietic cell transplants irrespective of detectable cytomegalovirus infection. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 11: 890-902.

200. Tormo, N., C. Solano, I. Benet, J. Nieto, R. de la Camara, A. Garcia-Noblejas, M. A. Clari, M. Chilet, J. Lopez, J. C. Hernandez-Boluda, M. J. Remigia, and D. Navarro. 2010. Kinetics of cytomegalovirus (CMV) pp65 and IE-1-specific IFNgamma CD8+ and CD4+ T cells during episodes of viral DNAemia in allogeneic stem cell transplant recipients: potential implications for the management of active CMV infection. *J Med Virol* 82: 1208-1215.

201. Solano, C., I. Benet, M. A. Clari, J. Nieto, R. de la Camara, J. Lopez, J. C. Hernandez-Boluda, M. J. Remigia, I. Jarque, M. L. Calabuig, A. Garcia-Noblejas, J. Alberola, A. Tamarit, C. Gimeno, and D. Navarro. 2008. Enumeration of cytomegalovirus-specific interferongamma CD8+ and CD4+ T cells early after

allogeneic stem cell transplantation may identify patients at risk of active cytomegalovirus infection. *Haematologica* 93: 1434-1436.

202. Tormo, N., C. Solano, I. Benet, M. A. Clari, J. Nieto, R. de la Camara, J. Lopez, N. Lopez-Aldeguer, J. C. Hernandez-Boluda, M. J. Remigia, A. Garcia-Noblejas, C. Gimeno, and D. Navarro. 2010. Lack of prompt expansion of cytomegalovirus pp65 and IE-1-specific IFNgamma CD8+ and CD4+ T cells is associated with rising levels of pp65 antigenemia and DNAemia during pre-emptive therapy in allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant* 45: 543-549.

203. Gratama, J. W., R. A. Brooimans, B. van der Holt, K. Sintnicolaas, G. van Doornum, H. G. Niesters, B. Lowenberg, and J. J. Cornelissen. 2008. Monitoring cytomegalovirus IE-1 and pp65-specific CD4+ and CD8+ T-cell responses after allogeneic stem cell transplantation may identify patients at risk for recurrent CMV reactivations. *Cytometry B Clin Cytom* 74: 211-220.

204. Morita-Hoshi, Y., Y. Heike, M. Kawakami, T. Sugita, O. Miura, S. W. Kim, S. I. Mori, T. Fukuda, R. Tanosaki, K. Tobinai, and Y. Takaue. 2008. Functional analysis of cytomegalovirus-specific T lymphocytes compared to tetramer assay in patients undergoing hematopoietic stem cell transplantation. *Bone Marrow Transplant* 41: 515-521.

205. Suessmuth, Y., R. Mukherjee, B. Watkins, D. T. Koura, K. Finstermeier, C. Desmarais, L. Stempora, J. T. Horan, A. Langston, M. Qayed, H. J. Khoury, A. Grizzle, J. A. Cheeseman, J. A. Conger, J. Robertson, A. Garrett, A. D. Kirk, E. K. Waller, B. R. Blazar, A. K. Mehta, H. S. Robins, and L. S. Kean. 2015. CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRbeta repertoire. *Blood* 125: 3835-3850.

206. Odendahl, M., G. U. Grigoleit, H. Bonig, M. Neuenhahn, J. Albrecht, F. Anderl, L. Germeroth, M. Schmitz, M. Bornhauser, H. Einsele, E. Seifried, D. H. Busch, and T. Tonn. 2014. Clinical-scale isolation of 'minimally manipulated' cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease. *Cytotherapy* 16: 1245-1256.

207. Einsele, H., M. Kapp, and G. U. Grigoleit. 2008. CMV-specific T cell therapy. *Blood Cells Mol Dis* 40: 71-75.

208. Tormo, N., C. Solano, I. Benet, J. Nieto, R. de la Camara, J. Lopez, A. Garcia-Noblejas, B. Munoz-Cobo, E. Costa, M. A. Clari, J. C. Hernandez-Boluda, M. J. Remigia, and D. Navarro. 2011. Reconstitution of CMV pp65 and IE-1-specific IFN-gamma CD8(+) and CD4(+) T-cell responses affording protection from CMV DNAemia following allogeneic hematopoietic SCT. *Bone Marrow Transplant* 46: 1437-1443.

209. Clari, M. A., G. Aguilar, I. Benet, J. Belda, E. Gimenez, D. Bravo, J. A. Carbonell, L. Henao, and D. Navarro. 2013. Evaluation of cytomegalovirus (CMV)-specific T-cell immunity for the assessment of the risk of active CMV infection in non-immunosuppressed surgical and trauma intensive care unit patients. *J Med Virol* 85: 1802-1810.

210. Loeth, N., K. Assing, H. O. Madsen, L. Vindelov, S. Buus, and A. Stryhn. 2012. Humoral and cellular CMV responses in healthy donors; identification of a

frequent population of CMV-specific, CD4+ T cells in seronegative donors. *PloS one* 7: e31420.

211. Hegde, N. R., C. Dunn, D. M. Lewinsohn, M. A. Jarvis, J. A. Nelson, and D. C. Johnson. 2005. Endogenous human cytomegalovirus gB is presented efficiently by MHC class II molecules to CD4+ CTL. *The Journal of experimental medicine* 202: 1109-1119.

212. Long, H. M., O. L. Chagoury, A. M. Leese, G. B. Ryan, E. James, L. T. Morton, R. J. Abbott, S. Sabbah, W. Kwok, and A. B. Rickinson. 2013. MHC II tetramers visualize human CD4+ T cell responses to Epstein-Barr virus infection and demonstrate atypical kinetics of the nuclear antigen EBNA1 response. *The Journal of experimental medicine* 210: 933-949.

213. Reddehase, M. J. 2002. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. *Nature reviews. Immunology* 2: 831-844.

214. Bourgault, I., A. Gomez, E. Gomard, F. Picard, and J. P. Levy. 1989. A virus-specific CD4+ cell-mediated cytolytic activity revealed by CD8+ cell elimination regularly develops in uncloned human antiviral cell lines. *Journal of immunology* 142: 252-256.

215. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *The Journal of experimental medicine* 203: 2865-2877.

216. Crompton, L., N. Khan, R. Khanna, L. Nayak, and P. A. Moss. 2008. CD4+ T cells specific for glycoprotein B from cytomegalovirus exhibit extreme conservation of T-cell receptor usage between different individuals. *Blood* 111: 2053-2061.

217. Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones. 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 8: 379-385.

218. Gamadia, L. E., R. J. Rentenaar, P. A. Baars, E. B. Remmerswaal, S. Surachno, J. F. Weel, M. Toebes, T. N. Schumacher, I. J. ten Berge, and R. A. van Lier. 2001. Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. *Blood* 98: 754-761.

219. van Leeuwen, E. M., J. J. Koning, E. B. Remmerswaal, D. van Baarle, R. A. van Lier, and I. J. ten Berge. 2006. Differential usage of cellular niches by cytomegalovirus versus EBV- and influenza virus-specific CD8+ T cells. *Journal of immunology* 177: 4998-5005.

220. Wherry, E. J. 2011. T cell exhaustion. *Nature immunology* 12: 492-499.

221. Cicin-Sain, L., A. W. Sylwester, S. I. Hagen, D. C. Siess, N. Currier, A. W. Legasse, M. B. Fischer, C. W. Koudelka, M. K. Axthelm, J. Nikolich-Zugich, and L. J. Picker. 2011. Cytomegalovirus-specific T cell immunity is maintained in immunosenescent rhesus macaques. *Journal of immunology* 187: 1722-1732.

222. Sester, U., D. Presser, J. Dirks, B. C. Gartner, H. Kohler, and M. Sester. 2008. PD-1 expression and IL-2 loss of cytomegalovirus- specific T cells correlates with viremia and reversible functional anergy. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 8: 1486-1497.

223. Terrazzini, N., M. Bajwa, S. Vita, E. Cheek, D. Thomas, N. Seddiki, H. Smith, and F. Kern. 2014. A novel cytomegalovirus-induced regulatory-type T-cell subset increases in size during older life and links virus-specific immunity to vascular pathology. *The Journal of infectious diseases* 209: 1382-1392.

224. Tovar-Salazar, A., J. Patterson-Bartlett, R. Jesser, and A. Weinberg. 2010. Regulatory function of cytomegalovirus-specific CD4+CD27-CD28- T cells. *Virology* 398: 158-167.

225. Bolovan-Fritts, C. A., R. N. Trout, and S. A. Spector. 2007. High T-cell response to human cytomegalovirus induces chemokine-mediated endothelial cell damage. *Blood* 110: 1857-1863.

226. Bolovan-Fritts, C. A., and S. A. Spector. 2008. Endothelial damage from cytomegalovirus-specific host immune response can be prevented by targeted disruption of fractalkine-CX3CR1 interaction. *Blood* 111: 175-182.

VIII) ERRATA SHEET

The thesis explores the T cell response to CMV in allogeneic hematopoietic stem cell transplant recipients using flow cytometric analysis of blood samples. In general it is a reasonable study although the analysis of CD8 responses is somewhat lacking in novelty.

Tal y como indica el revisor, son muy numerosos los estudios de monitorización de linfocitos T-CD8$^+$ específicos de CMV en pacientes sometidos a trasplante alogénico de precursores hematopoyéticos (alo-TPH), pero todo ellos están realizados mediante la cuantificación de las células específicas bien mediante el marcaje con tetrámeros o bien determinando la respuesta de las mismas *in vitro* tras el estímulo antigénico. En nuestro estudio nos propusimos incorporar a la práctica clínica, la posibilidad de cuantificar las células CD8$^+$ específicas frente a CMV con nuevos multímeros desarrollados recientemente y que "*a priori*" ofrecen ciertas ventajas sobre los tetrámeros convencionales.

Para ello se ha realizado un estudio exhaustivo sobre las ventajas e inconvenientes desde el punto de vista técnico de dos multímeros (pentámero y estreptámero. Inicialmente, según los resultados obtenidos hemos demostrado que el estreptámero (ST) es mejor que el pentámero para incorporarlo en la monitorización clínica de la respuesta inmune en estos pacientes ya que los resultados obtenidos son más específicos. De esta forma seleccionamos esta tecnología para realizar el estudio de monitorización en nuestros pacientes ya que no existen datos previos de la utilización de los ST en la monitorización de la respuesta inmune frente a CMV en pacientes sometidos a alo-TPH. Además, el estreptámero ofrece la posibilidad de ser utilizado en inmunoterapia adoptiva siendo uno de los objetivos de nuestro grupo a desarrollar en un futuro próximo.

- Schmitt, A., T. Tonn, D. H. Busch, G. U. Grigoleit, H. Einsele, M. Odendahl, L. Germeroth, M. Ringhoffer, S. Ringhoffer, M. Wiesneth, J. Greiner, D. Michel, T. Mertens, M. Rojewski, M. Marx, S. von Harsdorf, H. Dohner, E. Seifried, D. Bunjes, and M. Schmitt. 2010. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion* 51: 591-599.
- Neudorfer, J., B. Schmidt, K. M. Huster, F. Anderl, M. Schiemann, G. Holzapfel, T. Schmidt, L. Germeroth, H. Wagner, C. Peschel, D. H. Busch, and H. Bernhard. 2007. Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* 320: 119-131
- https://www.clinicaltrials.gov/ct2/NCT01077908/ or NCT01220895 or NCT01220895

The analysis of CD4 responses is the main area of new work.

El apartado de la respuesta específica de CMV por parte de las CD4, como bien dice el revisor, es más novedoso ya que todos los estudios que se conocen hasta la fecha se basan en la determinación de la respuesta $CD4^+$ tras la estimulación antigénica. En esta parte del trabajo desarrollado en la Universidad de Birmingham hemos conseguido desarrollar tetrámeros para clase II y se han caracterizado las propiedades fenotípicas y funcionales de estas células $CD4^+$-CMV específicas.

The organisation of the thesis is reasonable although there are multiple errors in the use of English (too many to list them fully). I recognise that English is not the student's first language but I believe these errors need to be corrected to avoid misunderstanding of the text.

De acuerdo a los consejos del revisor, se ha procedido a la corrección del inglés.

The introduction is a reasonably comprehensive account of viral infections in transplant recipients, but lacks a general introduction to immunology and more specifically T cell biology. There is insufficient introduction to T cell receptors, antigen processing, HLA etc, all of which are relevant to the thesis and therefore in my opinion should be introduced in more detail.

Teniendo en cuenta las sugerencias del revisor se ha incorporado en la Introducción un apartado describiendo aspectos generales de inmunología, relacionados con nuestro estudio: linfocitos T y sus receptores, HLA y procesamiento antigénico.

The hypothesis stated in the thesis has not formally been tested since the study is a retrospective one, rather than a prospective study seeking to determine whether immunological monitoring of CMV-specific immunity can predict CMV reactivations and help guide the choice of treatment.

Siguiendo el comentario del revisor, se ha modificado la hipótesis de trabajo, si bien no es un estudio retrospectivo sino prospectivo.

Throughout the study, immune responses to CMV are explored with very few (often one) multimeric reagents, and therefore are restricted to analysing T cell responses to just one or two epitopes. Can this be expected to give a true indication of the immune response to CMV in every transplant recipient? Equally can it be expected to determine the protective level required in all individuals to prevent CMV reactivations? What about responses to other epitopes and how should responses be monitored in people who are HLA A2 negative? All of this should be discussed. The sensitivity and specificity analysis was conducted with multimer reagents targeting a single epitope-specificity. Is this a fair test? Do these results

represent reagents that target other epitopes and HLA restrictions. Again, at the very least this issue should be discussed.

En la Discusión hemos incluido un párrafo en el que se discute que nuestros resultados del análisis técnico de ambos multimeros no pueden extrapolarse al estudio con otros multimeros con otras restricciones HLA ya que la avidez y afinidad de cada uno de ellos puede depender de la estructura química. También se ha justificado porque se ha elegido realizar el estudio en pacientes con restricción HLA-A*02:01 ya que de todas las restricciones HLA relacionadas con la presentación del CMV es la más frecuente en nuestra población.

I am concerned that there is little consideration of multiple comparisons. When conducting the statistical analyses and seeking for significant differences/correlations, a p value of 0.05 will not necessarily indicate significance if 20 comparisons have been made to find one that gives this value (since this p value means that such a difference could have occurred by chance on 1/20 occasions). This point should be discussed, as well as how future experiments should aim to test the hypotheses generated by some of the current data.

En este estudio sólo 6 variables clínicas han sido relacionadas con la reactivación del CMV y por lo tanto, no se considera necesario realizar ajustes de comparaciones múltiples. Por otro lado, no es el objetivo de nuestro estudio porque, tal y como se comenta en la discusión, ya existen muchos estudios relacionando dichas variables con la reactivación de CMV analizando un mayor número de pacientes.

The discussion chapter is more a summary of the results than a reflection on how the data compare with published studies and what it all means. There is some mention of other studies that give similar or contrasting results but little attempt to explain why others might have found a different result. More discussion is required on these points and others (e.g. what about using ST reagents to sort T cells for adoptive therapy? How is it best to determine the "protective cell number against CMV infection"? Expand upon why DYS-specific T cells differ in phenotype compared with AGI and LLQ-specific effectors. How might tetramers be used to study/sort CD4 responses in transplant recipients and what results might you expect?

De acuerdo a las sugerencias del revisor, el apartado de discusión se ha modificado intentando explicar las diferencias que nosotros observamos respecto otros investigadores así como incluido la utilización del ST en inmunoterapia adoptiva. También se ha dado una posible explicación a las diferencias observadas entre las distintas células T CD4+ específicas y cómo se podría incorporar el uso de tetrámeros en la monitorización de los pacientes sometidos a alo-TPH.

Page 15-table 2 there is an error in the reside numbers listed for the DR52-restricted epitope

Se ha corregido el error (509-523)

Page 17 – says CD45- when it should read CD45RA-

Se ha corregido.

Page 47 – why were the PM and ST reagents used at the concentrations stated? Had this been optimised in a titration experiment beforehand? If so show the data.

Las concentraciones y condiciones de marcaje de los multímeros (PM y ST) fueron optimizadas previamente en nuestro laboratorio (unpublished data). No se ha realizado en esta tesis por lo que no se añaden los resultados.

What is the relative cost of using PM vs ST reagents? This is a factor that should be discussed when considering which is the most appropriate to use for routine clinical monitoring.

Tal y como señala el revisor, en la elección de estos multímeros para su estudio y su posible incorporación en la monitorización de la respuesta inmune frente a CMV en pacientes que han sido sometidos a trasplante alogénico de precursores hematopoyéticos (alo-TPH), no sólo se tuvieron en cuenta las semejanzas o diferencias estructurales de los mismos sino también su coste. El PM tiene un coste similar al tetrámero, utilizado en muchos estudios, mientras que el ST tiene un coste menor, por lo que, no sólo ofrece ventajas técnicas sino también económicas respecto al PM (página 114).

Page 50 – "Detection of IFNgamma secreting cells by pentamer+ cells". In this protocol might not prelabelling the T cells with the pentamer at least partially inhibit their ability to then respond to the target peptide?

En el presente estudio realizamos el marcaje de la técnica siguiendo las instrucciones del kit utilizado (IFNγ Secretion Assay-Detection Kit, Miltenyi Biotec) y observamos diferencias respecto a la producción de IFNγ y la técnica de ICS. Tal y como nos comenta el revisor, el hecho de marcar con el pentámero previamente a la estimulación con el antígeno podría ser una posible explicación de las diferencias que observamos en la producción de IFNγ mediante ambas técnicas. Este comentario se ha incluido en la discusión. Es por lo tanto conveniente realizar futuros estudios para poder optimizar esta técnica que puede tener una gran utilidad para evaluar la funcionalidad de las células específicas del CMV.

Page 51 – "CMV-antigen detection in whole blood" – this is a misleading title because in fact it describes a serological assay which is detecting antibodies rather than antigen.

Se ha cambiado el título, ya que el revisor está en lo cierto y lo que detectamos son anticuerpos en el suero del individuo y no el antígeno de CMV "Anti-CMV antibody detection in whole blood".

Page 51 – give more details on the standard curve (what is it and how was it prepared)

Se ha explicado con más detalle la preparación de la curva estándar.

Page 55 – reference required for the MLA 144 cell line

Se ha añadido la referencia de la línea cellular MLA144.

Page 55 – LCLs were pulsed or labelled with peptide, not "stimulated"

Se ha modificado.

Page 55 – "50ul of the supernatant of each clone" – I assume you mean 50ul of cell suspension of each clone?

Se ha corregido puesto que es suspensión celular y no el sobrenadante.

Page 55 "negative and antigen-specific" – the meaning of this phrase is unclear

Se ha explicado correctamente a qué se refiere en esta frase para evitar confusiones.

Page 61 – comparing donors 20 and 29 in table 8 suggests PM are twice as sensitive as ST. Discuss.

En este análisis estamos evaluando la repetitividad del multímero utilizado, PM o ST, para lo cual se utilizaron 12 muestras diferentes. Los sujetos 20 y 29 fueron utilizados para evaluar tanto PM como ST, pero el análisis se realizó en momentos diferentes, por lo que el porcentaje de células específicas no tiene por qué coincidir con ambos multímeros. De ahí las diferencias encontradas entre ambos multímeros y que el revisor ha señalado. Es como si se tratara de sujetos diferentes. Para evitar confusiones y no cambiar la nomenclatura se ha añadido un comentario al pie de la tabla 8.

Page 63 – table 9 – what were the background levels of non-specific staining seen with these reagents (eg in CMV seronegative donors)?

El 'background' observado con cada multímero está explicado en la tabla 10.

Page 64 – the same sentence is repeated twice on this page.

Se ha eliminado la frase que estaba repetida: 'The median MFI values of the undiluted fraction of CMV-specific $CD8^+$ T cells were 19255 (10067 - 41913) and 8191 (5700 - 17127) for ST and PM respectively'.

Page 65 – figure 11 – how were the gates set that determine positively stained CMV-specific cells? There appear to be intermediate levels of staining for some T cells with both PM and especially ST reagents – why not include these as positive if they are above background?

Atendiendo a la pregunta del revisor, los gateos se fijaron definiendo clusters positivos. La población dispersa que se observa como positiva no se incluyó ya que no es una población clara y definida y puede ser debida a la inespecificidad del multímero, siendo mayor en el PM que en el ST.

Page 90 – it is important to remember that a T cell is not necessarily "non functional" because it does not make IFNgamma, it may yet make a different cytokine.

Estamos de acuerdo con el revisor en que el hecho de no producir IFN□ no necesariamente significa que la célula no sea funcional. Sin embargo, nosotros nos centramos en la producción de esta citoquina ya que la gran mayoría de estudios relacionados con la respuesta inmune frente a CMV estudian IFN□ (1,2). Además, un estudio realizado por Ghanekar y colaboradores, demuestra que la respuesta por parte de los linfocitos CD8+ T citotóxicos correlaciona significativamente con la producción de IFN□ tras estimulación antígeno específica y puede ser utilizado para estudiar la funcionalidad de las mismas (3). IFN□ es secretado por las células CD8+ citotóxicas específicas de CMV y está asociada con la infección por CMV. Por otro lado, existe un kit comercial para poder ser utilizado a escala clínica, conocido como QuantiFERON-CMV, donde se mide la producción de IFN□ tras estimulación con 23 epítopos de CMV con distintas restricciones alélicas que cubren más del 95% de la población (4). Además, el kit utilizado para medir la producción de citoquinas por parte de las células específicas de CMV (Miltenyi) sólo ofrecía la posibilidad de detectar directamente IFN□, para medir otras citoquinas era necesario combinar más de un kit.

1. Lilleri, D., G. Gerna, C. Fornara, A. Chiesa, G. Comolli, M. Zecca, and F. Locatelli. 2009. Human cytomegalovirus-specific T cell reconstitution in young patients receiving T cell-depleted, allogeneic hematopoietic stem cell transplantation. *The Journal of infectious diseases* 199: 829-836
2. Lilleri, D., P. Zelini, C. Fornara, G. Comolli, M. G. Revello, and G. Gerna. 2009. Human cytomegalovirus-specific CD4+ and CD8+ T cell responses in primary infection of the immunocompetent and the immunocompromised host. *Clin Immunol* 131: 395-403

3. Ghanekar, S. A., L. E. Nomura, M. A. Suni, L. J. Picker, H. T. Maecker, and V. C. Maino. 2001. Gamma interferon expression in CD8(+) T cells is a marker for circulating cytotoxic T lymphocytes that recognize an HLA A2-restricted epitope of human cytomegalovirus phosphoprotein pp65. *Clinical and diagnostic laboratory immunology* 8: 628-631
4. Tey, S. K., G. A. Kennedy, D. Cromer, M. P. Davenport, S. Walker, L. I. Jones, T. Crough, S. T. Durrant, J. A. Morton, J. P. Butler, A. K. Misra, G. R. Hill, and R. Khanna. 2013. Clinical assessment of anti-viral CD8+ T cell immune monitoring using QuantiFERON-CMV(R) assay to identify high risk allogeneic hematopoietic stem cell transplant patients with CMV infection complications. *PloS one* 8: e74744

Page 92 – fig 28 A and B – was a paired analysis used here?

Agradecemos al revisor esta apreciación y se ha realizado el test estadístico pareado apropiado (Wilcoxon).

Page 95 – "decreasing amounts of CD4 T cell clones" – by how much were they diluted? What were the predicted % positives and how well did the results correlate with these predictions?

Tal y como el revisor ha comentado se ha explicado cómo se realizaron las diluciones de los clones de células T CD4, así como cuáles eran los valores teóricos que se esperaban y la correlación de lo obtenido con lo esperado.

Page 97 – fig 31 – how many seronegative donors were studied and what was the range of responses seen with these donors? Show a graph of % tetramer positive cells seen with CMV seronegative donors vs seropositive donors so we can see the relative spread of responses in these two groups.

Desafortunadamente no se ha hecho el marcaje de los tetrámeros en sujetos seronegativos para CMV. Realizar dicho estudio podría ser de gran ayuda para determinar el punto de corte (cut-off) de la población positiva de la negativa. No obstante esto puede variar entre sujetos y hay que seleccionar cada caso. Sin embargo, no es posible realizar este marcaje ya que el estudio fue realizado durante la estancia en Birmingham y actualmente no tenemos los tetrámeros para realizar este marcaje y poder hacer el gráfico que muestre la diferencia entre sujetos positivos y negativos que el revisor comenta.

Los marcajes con los tetrámeros fueron considerados como positivos únicamente cuando se identificaba un cluster claro y bien definido. Los sujetos negativos que aparecen en la figura 31 son los que se utilizaron para el estudio de sensibilidad de cada clon de células T CD4 para dar una idea de la diferencia entre sujetos seropositivos y negativos para el CMV.

Page 98 – fig 32 there is no need for the split y axis in this figure

El eje de la figura se ha dividido para poder ver más claramente los puntos y que no queden tan juntos. No se ha modificado.

Page 98 and beyond – when characterising the tetramer positive population, there is a danger that results may include relatively few events and therefore the data could be unreliable. What was the minimum number of events used to determine a result and below which data were considered inadmissible?

Esta puntualización del revisor es una cuestión bastante discutida hoy en día en el ámbito de la citometría de flujo ya que existen diferentes criterios para definir la población positiva. Como bien hemos comentado anteriormente, en nuestros análisis se ha seguido el criterio de seleccionar la población tetrámero-específica como un cluster positivo claro y bien definido. No se ha establecido ningún número mínimo de células tetrámero positivas necesario para considerar el resultado como positivo. Sí que, en todos los sujetos analizados, al menos 300,000 linfocitos vivos fueron adquiridos (single life lymphocytes). Analizando el número de eventos positivos, $CD4^{+}$tetrámero^{+}, en aquellos sujetos en los que se identificó un cluster definido como positivo, el número mínimo de eventos adquirido fue de 40.

Page 99 – fig 33 B – should y axis title include CCR7-ve?

No debe incluir CCR7- ya que se está hablando de la frecuencia de expresión de CD45RA en las células específicas de DYS, AGI o LLQ, no de CD45+CCR7-.

Page 100 – differences found in the DYS-specific T cells vs the AGI and LLQ –specific T cells – might these be explained by a disproportionate number of old or young donors respectively studied for each of these responses?

En nuestro caso concreto, no hemos observado grandes cambios en relación a la edad. Sin embargo, puede que sea cierto lo que el revisor señala, si bien no se ha hecho el estudio comparativo de las células específicas de DYS, AGI o LLQ dentro de cada grupo de edad por la imposibilidad de encontrar el mismo número de sujetos para cada condición.

Page 102 – fig 36 legend should say CD27-CD28-

Se ha corregido.

Page 105 – how did NKG2D expression compare on the DYS-specific T cells vs the AGI and LLQ –specific T cells? The data should already be available.

Se han incluido las frecuencias de expresión de NKG2D en las células específicas de CMV en función del epítopo (DYS, AGI y LLQ) (figura 40, pag.106).

Page 107 – why is PD1 expression reduced on DYS-specific T cells vs the AGI and LLQ – specific T cells? Discuss.

Agradecemos esta puntualización del revisor. Los resultados obtenidos de la expresión de PD1 son contrarios a lo esperado, puesto que al ser un marcador de 'agotamiento' celular (del inglés T-cell exhaustion), era de esperar que se expresara en mayor proporción en aquellas células que se encuentran en un estadío de mayor diferenciación. En nuestro caso, las células específicas de CMV que reconocen el péptido DYS están más diferenciadas que las que reconocen los péptidos AGI o LLQ, presentando un fenotipo memoria efector, ausencia de las moléculas coestimuladoras CD27 y CD28 y un aumento de la expresión de CD57. Estudios preliminares realizados a posteriori mediante selección de células PD1+ y PD1- específicas de CMV demuestran que no existen diferencias ni desde el punto de vista funcional ni de expresión génica. Son necesarios realizar futuros estudios para explicar estos resultados.

Page 108 – fig 43 – how does expression of CX3CR1 compare on the CMV-specific T cells with other effector memory T cells in these donors? Is this a CMV-specific T cell phenotype or a marker of effector memory T cells in these donors?

En realidad, no tenemos en el panel en el que miramos la expresión de CX3CR1, ningún marcador que nos permita distinguir entre las distintas subpoblaciones celulares por lo que no podemos ver y comparar la expresión de CX3CR1 en otras células memoria efectora, como el revisor sugiere. Tampoco podemos determinar, mediante este estudio si la expresión de CX3CR1 está asociado con la infección por CMV o si es un marcador de las células memoria efectoras. Sin embargo, un estudio realizado por Hertoghs y colaboradores (1), sugiere que CX3CR1 parece ser un marcador de las células memoria efectoras $CD8^+$ específicas de CMV, ya que no se observa este marcador ni en células específicas de EBV o influenza. Un estudio realizado por Sacre y colaboradores demuestra que pacientes con VIH tenían presencia de células T CD4+ específicas de CMV que expresaban CX3CR1 tras estimulación antigénica (2).

Se han realizado estudios posteriores, no incluidos en esta tesis, en sujetos seropositivos y seronegativos de CMV en los cuales se marcó con CD4, CD8, CD28 y CX3CR1. La expresión de CX3CR1 se detectó únicamente en sujetos CMV seropositivos, siendo mayoritariamente CD28 negativos. Por lo que podríamos decir que la expresión de CX3CR1, es un marcador de fenotipo de las células específicas de CMV más que de células memoria efectoras. Cabe resaltar que estas células específicas de CMV son en su mayoría memoria efectoras.

1. Hertoghs, K. M., P. D. Moerland, A. van Stijn, E. B. Remmerswaal, S. L. Yong, P. J. van de Berg, S. M. van Ham, F. Baas, I. J. ten Berge, and R. A. van Lier. 2010. Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. *The Journal of clinical investigation* 120: 4077-4090

2. Sacre, K., P. W. Hunt, P. Y. Hsue, E. Maidji, J. N. Martin, S. G. Deeks, B. Autran, and J. M. McCune. 2012. A role for cytomegalovirus-specific CD4+CX3CR1+ T cells and cytomegalovirus-induced T-cell immunopathology in HIV-associated atherosclerosis. *Aids* 26: 805-814